Centro de Investigación en Alimentación
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EFECTOS A NIVEL CELULAR Y MOLECULAR DE LA INGESTIÓN DE SOYA ENZIMATICAMENTE MODIFICADA PARA LA RECUPERACION DE RATAS DESNUTRIDAS

Por

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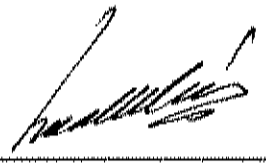
Los miembros del comité designado para revisar la tesis de Abraham Wall Medrano, la han encontrado satisfactoria y recomiendan sea aceptada como requisito parcial para obtener el grado de Doctorado en Ciencias.



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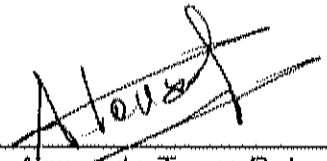

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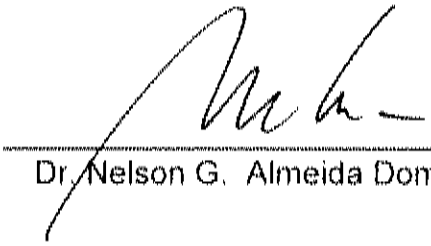

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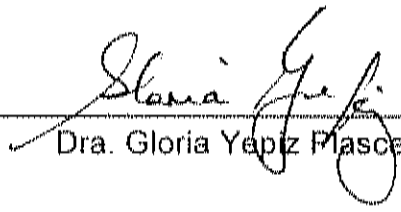

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Dr. Alfonso A. Gardea Béjar
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DEDICATORIAS

¡Qué lástima
que yo no pueda cantar a la usanza
de este tiempo lo mismo que los
poetas de hoy cantan!
¡Qué lástima
que yo no pueda entonar con una voz
engolada
esas brillantes romanzas
a las glorias de la patria!
¡Qué lástima
que yo no tenga una patria!

Sé que la historia es la misma, la
misma siempre, que pasa
desde una tierra a otra tierra, desde
una raza
a otra raza,
como pasan
esas tormentas de estío desde esta a
aquella comarca
¡Qué lástima
que yo no tenga comarca,
patria chica, tierra provinciana!

Debí nacer en la entraña
de la estepa castellana
y fui a nacer en un pueblo del que no
recuerdo nada
pasé los días azules de mi infancia en
Salamanca,
y mi juventud, una juventud sombría,
en la Montaña.
después .. ya no he vuelto a echar el
ancla,
y ninguna de estas tierras me levanta
ni me exalta
para poder cantar siempre en la
misma tonada
al mismo río que pasa
rodando las mismas aguas,
al mismo cielo, al mismo campo y en
la misma casa.

¡Qué lástima que yo no tenga una
casa!
Una casa solariega y blasonada,
una casa
en que guardara,
a mas de otras cosas raras,
un sillón viejo de cuero, una mesa
apolillada
y el retrato de un mi abuelo que
ganara
una batalla.

¡Qué lástima
que yo no tenga un abuelo que
ganara
una batalla,
retratado con una mano cruzada
en el pecho, y la otra mano en el
puño de la espada!
Y, ¡qué lástima
que yo no tenga siquiera una espada!

Porque..., ¿qué voy a cantar si no
tengo ni una patria,
ni una tierra provinciana,
ni una casa
solariega y blasonada,
ni el retrato de un mi abuelo que
ganara
una batalla,
ni un sillón viejo de cuero, ni una
mesa, ni una espada?
¡Qué voy a cantar si soy un paria que
apenas tiene una capa!

Sin embargo ..
en esta tierra de España
y en un pueblo de la Alcarria
hay una casa
en la que estoy de posada
y donde tengo, prestadas,
una mesa de pino y una silla de paja
Un libro tengo también. Y todo mi
ajuar se halla
en una sala
muy amplia
y muy blanca
que está en la parte mas baja
y mas fresca de la casa
Tiene una luz muy clara
esta sala
tan amplia
y tan blanca ..
Una luz muy clara
que entra por una ventana
que da a una calle muy ancha
Y a la luz de esta ventana
vengo todas las mañanas

Aquí me siento sobre mi silla de paja
y venzo las horas largas
leyendo en mi libro y viendo cómo
pasa
la gente al través de la ventana.
Cosas de poca importancia
parecen un libro y el cristal de una
ventana
en un pueblo de la Alcarria,
y, sin embargo, le basta
para sentir todo el ritmo de la vida a
mi alma.

Que todo el ritmo del mundo por
estos cristales pasa
cuando pasan
ese pastor que va detrás de las
cabras
con una enorme cayada,
esa mujer apobiada
con una carga de leña en la espalda,
esos mendigos que vienen
arrastrando sus miserias de Postrana
y esa niña que va a la escuela de tan
mala gana.

¡Oh, esa niña! Hace un alto en mi
ventana
siempre y se queda a los cristales
pegada
como si fuera una estampa
¡Qué gracia
tiene su cara
en el cristal aplastada
con la barbilla sumida y la naricilla
chata!

Yo me río mucho mirándola
y la digo que es una niña muy
guapa ..
Ella entonces me llama ¡tonto!, y se
marcha
¡Pobre niña! Ya no pasa
por esta calle tan ancha
caminando hacia la escuela de muy
mala gana,
ni se para
en mi ventana,
ni se queda a los cristales pegada
como si fuera una estampa.

Que un día se puso mala,
muy mala
y otro día doblaron por ella a muerto
las campanas.
Y en una tarde muy clara,
por esta calle tan ancha,
al través de la ventana,
vi como se la llevaban
en una caja muy blanca ..

En una caja
muy blanca
que tenía un cristalito en la tapa,
Por aquel cristal se la veía la cara
lo mismo que cuando estaba
pegadita al cristal de mi ventana...
Al cristal de esta ventana
que ahora me recuerda siempre el
cristalito de
tan blanca.
Todo el ritmo de la vida pasa
por este cristal de mi ventana...
Y la muerte también pasa!

¡Qué lástima
que no pudiendo cantar otras
hazañas,
porque no tengo una patria
ni una tierra provinciana,
ni una casa
solariega y blasonada,
ni el retrato de un mi abuelo que
ganara
una batalla
ni un sillón viejo de cuero, ni una
mesa, ni una espada,
y soy un paria
que apenas tiene una capa ..
venga, forzado, a cantar cosas de
poca importancia!

LEON FELIPE

Por enseñarme a amar a la humanidad y a la vida. Esto va para ustedes **Familia**, **Ana María**, **Arturo** y donde estés **Papá**.

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Quiero ganar mi verso,
este verso;
y quiero
que vaya quedo,
raudo y sereno
como un dardo certero
al corazón del pueblo
de todos los pueblos...
al corazón del Universo
León Felipe

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CONTENIDO

INTRODUCCIÓN

La desnutrición es una de las causas mas importantes de mmbi- mmtalidad infantil (1). A los hospitales ingresan nifios desnutridos por diversas causas, principalmente por la falta de alimentos o por infecciones recurrnntes. En otras ocasiones, los ni11os ingresan por distintas patologlas pero con un estado nutricio aceptable y una vez en el 1·1ospital, desarrollan sIntoinas de desnutrición. El núrm ro de niños ingresados por desnutrición va en aumento mientras que la desnutrición intra· hospitalaria se controla cada vez mejor (2). Aún cuando la desnutrición Infantil puede tem r distintos orlgenes y puede ir desde formas leves a graves (e.g. kwashiorkor y marasmo), en la mayorla de los casm, hay disfunción Jastrointestinal (3,4). La incapacidad para asimilar de manera correcta los alimentos requiere una asistencia nutricional especial e inmediata dentro de los hospitales, la cual algunas veces resulta inadecuada (5,6).

En la rehabilitación nutricir.inal 1fa infantes desnutridos se han usado diversas formas de nitrógeno dietario, Se han evaluado tanto fórmulas poliméricas basadas en protelnas Intactas (7,8), sernielementales u oligoméricas y elementales o rnonoméricas basadas en aminot. cidos libres (9). Aunque la OMS (1 O) recomienda tratar intral1ospltalariamente la desnutrición infantil con prolt,Inas lácteas, se han utilizado también con este propósito otras fuentes proteicas como la soya, desde l1ace 75 af1os ('11). Las primeras fórmulas enterales a base de soya se preparaban con la harina obtenida al extraerle el aceite al grano: posteriormente S(1 lm usado la protelna aislada de soya (SPI), fortificando el producto con metionina libre (12). Debido a la buena respuesta biológica a formulaciones basadas en SPI, la Academia A1111ricana de PediaIrla (13) las ha recomendado como altemativa para infantes que no pueden ser alimentados al pecho o con fórmulas a base de protetnas likteas,

El procesamiento industrial para obtener la protelna aislada de soya reduce pero no inhibe por completo la actividad de factores antifisiológicos y alergenos naturales en la soya (14, 15). Este tipo de compuestos, limita no solo la calidad nutricia (16, 17) sino que pueden ser perjudiciales para el desarrollo flsico del infante (18). De hecho, en algunos casos los ni1ios con gastroenteritis aguda pueden desarrollar enteropatias relacionadas al consumo de soya ('19).

Una solución tecnológica al problema anterior, 1·1asido la hidrólisis parcial de proteínas de soya, ya que varios de los factores mencionados son de naturaleza proteica. Otros investigadores y nosotros mismos (20,2·1) hemos propuesto ademtis de la hidrólisis, la ultrafiltración para limitar el tamai,o peptldico, lo cual reduce la alergenicidad y aumenta la posibilidad de absorción intestinal. Asl mismo, dicha propuesta involucra un paso de sintesis enzimática para modificar la composición de aminotwidos, en especial rnetionina, lo cual mejora la biodisponibilidad del aminoácido con respecto a su forma libre.

De acuerdo a dicha propuesta, hemos desarrollado una fórmula basada <n protelna de soya enzimáticarnente modificada (MSP), con una distribución de péptidt)S entre 1 y 1O kDa y enriquecida covalenternente con metionina. El valor nutricional de tal producto es superior al del aisl;3do de soya con lll(tionina libre, cuando se ha evaluado rn ratas adultas sanas (22) y en ratas jóvenes desnutridas (23). Sin embargo, la única ventaja de dicha fracción de protelna de soya modificada enzi111átican1ente sobre caselna, ingrediente común de las fórmulas enterales, es la reducción en alerg< nicidad.

La presente tesis pretende contribuir al conocimiento sobre el valor nutricional de MSP en la rehabilitación de la desnutrición. Los efectos buscados pueden encontrarse a nivel macro (respuesta biológica general), celular (estructura y proliferación celular) o molecular (expresión genética de

rnedíador is de crecimiento). Asi, se estudiaron los efectos de MSP fil todos estos nivelc1s, haciendo las comparaciones respedivas con dos pro\elnas de referencia (un aislado de soya comercial y caseina), con1únrnente usadas en la preparación de fórmulas enterales para infantes, Este estudio evalúa no solo la respuesta biológica general, sino el esll111ulo que el tipo y cantidad de la protelna dietaría administrada, ejerce sobre la restauración funcional de dos órganos de vital importancia duran\ ) la recuperación nutrici;.1: el l1lgado (intestino.

PLANTEAMIENTO DEL PROBLEMA EN ESTUDIO

Para reactivar el proceso proliferatlvo y funcionalidad de hlgado e intestino durante la recuperación de la desnutrición, son necesarias distintas sef\ales tanto exógenas (dieta) como endógenas (promotores de crecimiento). Uno de los eventos metabólicos en los que se integrnn todos estos aspectos es el de la biosintesis y degradación de poliaminas. Estas moléculas con carácter policati6nico son indispensables para la replicación del DNA (24), se encuentran distribuidas en todas las células (25) y son producidas a partir de aminoácidos precursores como la metionina y arginina **(Fig. 1,** pp. 2(:l). Su metabolismo depet1de de varias enzimas y protelnas reguladoras (24) cuya actividad se regula por est.lmulos hormonales, principalmente a nivel transcripclonal **(Tabla** 1, pp. 24). La concentración intracelular de las tres principales polian1inas (putrecina espermldim·l y esp('lrtnina) a su vez, produce sefíales dentro de la célula que retroregulan a las enzimas biosintéticas, transcripcional y post. traduccionalrnente **(Fig. 2,** pp. 27).

Además, las dos principales enzimas encargadas de la biosintesis de poliaminas (Ornitln· y S·adenosiltmitionina descarboxilasas) responden a variables dietarias como la cantidad y calidad de protelna (26,27). La regulación

tan estricta en la concentración de poliaminas intracelulares incide en la proliferación celular; por ello tras su depleción, se detiene el crecimiento por acción de proteínas específicas tales como la p53 y el NF-κB (28,29). Además, en una situación como la desnutrición, existe un estricto control entre la utilización de aminoácidos como combustibles o para crecimiento celular. Es por esto que, para lograr una recuperación nutricional eficiente de cada órgano desnutrido, se requiere de proteínas dietarias de alta calidad, que induzcan la transcripción de genes involucrados en la biosíntesis de poliaminas.

Por otra parte, la calidad de una proteína depende directamente de la eficiencia en su absorción intestinal y biodisponibilidad a tejidos periféricos como el hígado. Por ello, es probable que la forma, polimérica u oligomérica, en que esta sea suministrada y su contenido de aminoácidos, en especial arginina y metionina, activen de manera diferencial la expresión de genes del metabolismo de poliaminas.

METODOLOGÍA

Se realizaron tres bioensayos secuenciales. En el *primero*, 3 grupos de 6 ratas (*Rattus norvergicus*) de 21 días de edad fueron alimentadas por espacio de 14 días con tres dietas al 10% de proteína basadas en caseína (CAS), una mezcla de oligopéptidos (1-10 kDa) de soya con metionina covalentemente enlazada (MSP) y aislado de soya suplementado con suficiente metionina libre (SPI) hasta alcanzar el contenido de MSP. Este bioensayo sirvió para evaluar la respuesta biológica a las tres dietas en condiciones normales. En el *segundo*, 12 ratas de la misma edad fueron alimentadas durante 21 días con una dieta de caseína al 4% (CAS4%). Simultáneamente se alimentaron 4 ratas con una dieta al 18% (CAS18%) durante el mismo período. Con este bioensayo se pretendió establecer el mejor tiempo de inducción de desnutrición. Por último, en el

tercero, se alimentaron con CAS, MSP o SPI al 18% de la dieta, 3 grupos de ratas desnutridas (n=12 c/u), durante 21 días. Mientras que en el primer bioensayo los muestreos y sacrificio de animales se realizaron al final, en el segundo y tercero se realizaron muestreos aleatorios cada semana.

Primeramente se evaluó, por medio de indicadores macroscópicos, la respuesta biológica de ratas sanas y desnutridas, a las tres dietas experimentales mencionadas anteriormente (**Artículo 2**, pp. 29-56). La evaluación nutricional de MSP al 10% sobre las correspondientes basadas en CAS y SPI en las ratas sanas se sustentó en parámetros de calidad proteica, digestibilidad verdadera e ileal, urea y aminoácidos plasmáticos, así como amoniaco y urea urinarios. Durante el bioensayo de inducción de desnutrición (CAS 4%) y en las ratas desnutridas en recuperación (dietas al 18%) se evaluaron parámetros de calidad proteica, digestibilidad aparente y proteínas, urea y ácidos grasos plasmáticos. Además, se realizaron confirmaciones histológicas del grado de desnutrición durante el bioensayo de inducción por medio de tinciones (ácido periódico de Schiff y hematoxilina-eosina) en hígado e intestino.

Para las evaluaciones a nivel celular y molecular se colectaron el hígado, íleon y yeyuno de los animales de los bioensayos segundo y tercero, después de anestesia y sacrificio. Estos tejidos se homogeneizaron y congelaron individualmente, para después reunir alícuotas por grupo experimental y aislar el RNA total. Se cuantificaron los niveles de expresión por Transcripción reversa y amplificación por la reacción en cadena de la polimerasa (RT-PCR) y utilizando iniciadores específicos para los siguientes genes: Ornitín decarboxilasa (ODC), *S*-adenosilmetionina decarboxilasa (AdometDC), Espermidina sintasa (SPdS), Espermina sintasa (SPmS), Espermidina/Espermina-*N1*-acetiltransferasa, ODC antizima (OAZ) y su

inhibidor (OAZI). El factor nuclear alfa activado por proliferadores de peroxisomas (PPARα). La expresión individual de cada uno de estos genes fue normalizada a los valores correspondientes del gen constitutivo gliceraldehido-3-fosfato deshidrogenasa (GAPDH). Así mismo, se realizó en cortes histológicos de ileon y yeyuno, la inmunodetección de dos proteínas que estimulan la proliferación celular (antígeno nuclear de proliferación celular, PCNA) o el arresto de la misma (p53).

Todas las variables experimentales fueron evaluadas por dieta, grupo y estadio experimental por medio de análisis de varianza con una significancia $p<0.05$ y la prueba de Tukey-Kramer de comparación de medias. Así mismo, se realizaron análisis de correlación de Spearman (rs) para comparar los efectos de las distintas variables de acuerdo con los grupos experimentales.

RESULTADOS Y DISCUSIÓN

De acuerdo a los resultados descritos en el **Artículo 2** (pp. 29-56), en las ratas bien nutridas, MSP fue tan bueno como CAS pero mejor que SPI en términos de digestibilidad (verdadera e ileal) y su correspondiente calificación química (PDCAAS, PIDCAAS) y urea plasmática (**Tablas 1** y **2,** pp. 49 y 50). Aún cuando las ratas alimentadas con SPI consumieron menos proteína que aquellas alimentadas con CAS y MSP, todas presentaron el mismo balance nitrogenado y eficiencia proteica (**Fig. 1A**, pp. 55). Sin embargo, las ratas alimentadas con CAS excretaron mas urea y aquellas alimentadas con SPI más amoníaco en orina que las del grupo alimentado con MSP. La respuesta post-prandial de aminoácidos a las 2 h de ingestión fue superior en los animales alimentados con CAS que aquellos alimentados con las dietas de soya (**Tabla 3**, pp. 51). Sin embargo, la relación de aminoácidos no esenciales: esenciales

(NEAA:EAA) fue similar en los animales alimentados con SMP y CAS pero superior al encontrado para el grupo que consumió SPI.

En nuestro país, como en muchos otros, las formas de desnutrición energético proteica son resultado principalmente de una alimentación con bajo contenido y calidad proteica (30). Por eso el protocolo de inducción de desnutrición de este estudio pretendía generar un estado similar al de una población infantil desnutrida. La tercer semana de alimentación con una dieta baja en proteína (CAS4%), fue seleccionada con base en la respuesta biológica de los animales (**Fig. 1B** y **Tabla 4**, pp 55 y 52) y en los hallazgos histológicos (**Fig. 2**, pp. 56). Estos animales no presentaron balances de nitrógeno negativos y sostuvieron la utilización de proteína para crecimiento, aunque en una tasa menor que la de los animales bien nutridos (grupo CAS18%). Así, se seleccionó el período de 21 días de inducción de la desnutrición con base en algún grado de anorexia, bajos niveles de proteínas plasmáticas pero altos de ácidos grasos y una pérdida sustancial de organización celular en intestino y de reservas energéticas en hígado.

En las ratas desnutridas en recuperación dietaria, se pudo detectar un mejor efecto en la administración de soya modificada (MSP) en relación al encontrado para la soya intacta (SPI), particularmente en utilización de proteína para crecimiento (**Fig. 1C**, pp. 55). Sin embargo estas diferencias no fueron evidentes en comparación a los animales alimentados con CAS en términos de indicadores bioquímicos y calidad de proteína (**Tabla 5**, pp. 53) e incluso en restauración morfológica del hígado e intestino de éstas ratas (datos no mostrados). Con base en todos estos hallazgos, este primer estudio sugirió que el MSP puede ser una mejor opción que el aislado de soya pero igual a la caseína en la recuperación nutricional de organismos desnutridos.

En la búsqueda de un argumento que indicara diferencias en calidad nutricional entre MSP y CAS, se evaluó la respuesta celular y molecular de la rata desnutrida antes y durante la recuperación nutricional con estas dos fuentes proteicas (**Artículo 3**, pp. 57-86). Se ha demostrado que el aislado de soya en comparación a la caseína, provoca la expresión diferencial de varios genes involucrados en el crecimiento celular, cuando se administra al mismo nivel de proteína a animales bien nutridos (31). Sin embargo su deficiencia en metionina no promueve el crecimiento de las células tumorales ni su suplementación con metionina libre corrige este hecho (32).

Dado que MSP es igualmente digestible que CAS pero su contenido de arginina y metionina covalentemente enlazada es superior (**Tabla 2**, pp. 50), es de esperarse una respuesta diferencial en la activación de la biosíntesis de poliaminas y por ende en el crecimiento celular. Es por esto que se analizó, por medio de RT-PCR la acumulación de mRNA de la mayoría de los participantes del metabolismo de poliaminas (**Fig. 1,** pp. 26). La regulación de estos se da principalmente a nivel transcripcional (10, 26), con una participación muy activa en el proceso de proliferación celular. Así mismo, ya que los niveles intracelulares de las distintas poliaminas influyen en la transcripción y estabilización del proto-oncogen p53 (28,28) y que este último influye en la expresión (33) y participación durante el ciclo celular (34) del antígeno nuclear de proliferación celular (PCNA, por sus siglas en ingles), ambas proteínas fueron inmunodetectadas en los tejidos intestinales.

Previo a la rehabilitación con CAS o MSP, los animales desnutridos mostraron una disminución significativa en los niveles de mRNA para casi todos los participantes del metabolismo de poliaminas en los tres tejidos analizados. Como era de esperarse, el consumo de los dos aminoácidos precursores de la biosíntesis de poliaminas, metionina y arginina, fue superior en los animales

alimentados con MSP que en aquellos alimentados con CAS (**Tabla 2**, pp. 81). Esto posiblemente se debió a la activación inmediata de la S-adenosilmetionina decarboxilasa (AdometDC) en tejidos intestinales en los animales alimentados con MSP (**Tablas 3 y 4**, pp. 82 y 83). Los incrementos paulatinos en la expresión de los genes reguladores (OAZ y OAZI) de la ornitin decarboxilasa (ODC), aparentemente fueron dependientes de la forma de la proteína suministrada. Por su parte, el hígado mostró una respuesta distinta al de los tejidos intestinales, posiblemente a consecuencia del proceso de restauración entérica y liberación de aminoácidos dietarios a circulación sistémica (**Tabla 5**, pp. 84). Además, en el hígado de las ratas alimentadas con MSP, los niveles de mRNA de ODC correlacionaron con la ingestión de arginina, al igual que entre AdometDC y metionina.

Por su parte, los indicadores inmunohistoquímicos mostraron una posible dependencia de la forma en que se suministró la proteína dietaria y la presencia de la proteína p53. Esta última parece contener el estímulo de crecimiento en las ratas alimentadas con CAS pero no en aquellas alimentadas con MSP. Los hallazgos descritos en el **Artículo 3** permiten asegurar que, al menos en relación a los genes analizados, existen diferencias a nivel molecular tras la administración de MSP en comparación a CAS. Estos efectos también se tradujeron en estímulos diferenciales de crecimiento celular en el intestino de las ratas en recuperación.

Por otra parte, como se esquematiza en la **Fig. 3** (pp. 28), al factor de transcripción conocido como receptor alfa activado por proliferadores de peroxisomas (PPARα) se le ha involucrado recientemente con la regulación negativa de dos enzimas del metabolismo de poliaminas (35). La acción negativa de este factor de transcripción parece antagonizar la de otros factores como c-myc y C/EBPα en la regulación del metabolismo de poliaminas (**Tabla**

1, pp. 24). El PPARα es sensible a los patrones dietarios y se puede activar eficientemente en condiciones metabólicas similares a las encontradas en la desnutrición (36).

Por lo anterior, el **Articulo 4** (pp. 87-107) presentado en esta tesis se avoca al estudio del efecto de la administración de CAS y MSP en los niveles de mRNA de PPARα en hígado, yeyuno e ileon y su posible relación con los de la espermidina sintasa (SPmS) y espermidina/espermina-*N1*-acetiltransferasa (SSAT). La cuantificación de estos tres genes también se realizó por RT-PCR y se buscaron correlaciones con variables dietarias como la ingestión de proteína y energía. CAS y MSP resultaron igualmente capaces de estimular los niveles de expresión de PPARα durante todo el proceso de recuperación de la desnutrición. El nivel de proteína en la dieta tiene una influencia en los niveles de mRNA para PPAR α en los tres tejidos pero de una manera diferencial (**Fig. 1,** pp. 105). Mientras que, el nivel de mRNA de este factor de transcripción no tuvo relación con el de SPdS y SSAT en ninguno de los tejidos analizados durante la recuperación nutricional de los animales desnutridos (**Fig. 2**, pp. 106).

Además, los patrones de expresión de PPARα en hígado e ileon parecían tener relación con la utilización de nutrientes y su expresión en yeyuno. Por esto en este trabajo postulamos que, aún cuando los niveles de PPARα no tuvieron relación significativa con la expresión de SPdS y SSAT, la utilización gradual de los nutrientes luminales a nivel de yeyuno, provoca una movilización gradual de los mismos a tejidos periféricos e ileon dependiente de la restauración epitelial y funcional del yeyuno (**Fig. 3**, pp. 107).

CONCLUSIONES

Los efectos benéficos de la soya enzimáticamente modificada en la rehabilitación nutricional de organismos desnutridos radica en una asimilación elevada y su perfil de aminoácidos. El efecto, en comparación al de la proteína de soya intacta (SPI) es evidente desde el nivel global de respuesta biológica, mientras que las diferencias con la caseína, se detectan solo a nivel celular e incluso molecular. El soporte experimental ofrecido en este trabajo permite afirmar que esta fuente de proteína puede ser una buena alternativa para el tratamiento de la desnutrición o de algún otro desorden que comprometa la absorción intestinal de proteínas dietarias.

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The Polyamine's play: Novel actors, best performance and modifications of the old script

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ABSTRACT

New tools in molecular nutrition reveal new participants that modified the polyamine pathway scenario. Polyamine homeostasis, intracellular signaling and mobilization of new regulatory proteins are emerging as potential determinants of cell growth and differentiation. Understanding their inter-relationship may help to understand how cell moves toward different adaptation mechanisms to control polyamines in disease. Here, we review novel aspects in gene expression and action of polyamine pathway-related proteins and how they are controlled in healthy and altered physiological conditions. Facts about interaction and cross-talking between polyamines and other endogenous substrates are also discussed, particularly in liver and small intestine.

Keywords: Polyamines, malnutrition, signal transduction, gene regulation.

Actors on the scene: New script for the old and new ones.

Polyamines play key roles in several cellular processes like growth, differentiation and apoptosis (1,2). Elevated polyamine (namely putrecine, spermidine and spermine, **Figure 1**) synthesis is an initial and obligated event for cell proliferation and any disturbance that may arrest cell growth. Their polybasic and aliphatic character and binding capacity to negative ions, like DNA-phosphate moieties, leads to conformational changes in DNA grooves (3). These interactions are also dependent on specific sequences of nucleic acids that induce a conformation shifting from right-handed B form to left-handed Z form. This B to Z transition is a conditional event for DNA condensation (4) and gene expression (5). Higher number of carbon atoms and amido groups correlates with higher affinity of polyamines to DNA at different positions (3). Since the interactions occur by simple van der Waals bonds, the intracellular concentration of each polyamine must be tightly controlled.

Polyamines are absolutely required for proliferation (lower limit) and toxicity (upper limit) which dictates the stretch range of each polyamine within a specific cell type. In fact, it has been suggested that the polyamine content of an organ correlates with its metabolic activity (6). Since polyamines are widely distributed in the body and many processes are involved in their homeostasis, it is virtually impossible to observe a systemic and serum polyamine imbalances in healthy conditions (7).

In normal cells, individual concentration of each polyamine results from their uptake from extra-cellular sources, intracellular *de novo* synthesis, retro-conversion and degradation (2). Liver is capable to uptake blood free polyamines while gastrointestinal tissues can rapidly uptake dietary and gut bacterial-derived polyamines (6). The polyamine transport is an efficient energy requiring process that works against a concentration gradient, several of its components principally in the intestine have been reported (8). Although it has been recognized that gut absorption of luminal polyamines contributes significantly to the total polyamine body pool, the food-derived polyamines are restricted just to few food sources (6). Additionally, polyamine depletion in intestinal cells causes p53 expression (9) and stabilization and NF-κB activation (10) as well as a concomitant cell growth depletion. Therefore, if dietary polyamines are principally used for intestinal growth, the intracellular biosynthesis mechanisms are extremely important to supply peripheral tissues like liver.

In general, *de novo* synthesis of polyamines from amino acid represents the principal intracellular source of polyamines. The conditionally essential amino acid arginine (Arg) and the essential one, methionine (Met) are both needed for a right performance of the polyamine pathway (**Figure 1**). Although most of the dietary Arg and Met are metabolized at intestinal (40-52%) and

hepatic (≈ 50%) level, their metabolic fate is not restricted to polyamine production (11). Dietary Arg does not appear essential for the maintenance of free polyamine levels in adult animals, although it is indispensable for the compromised gut metabolism and repair (12,13). On the other hand, although Met has an important participation in biosynthesis of the first aminoacyl-tRNA for protein synthesis, may also be utilized in other pathways (14). Therefore, it is important for the correct activation of the polyamine pathway, a continuous intracellular biosynthesis of Arg, as well as enough exogenous supply of Met and Arg. In addition to Arg and Met supply to activate the polyamine pathway, their transformation to direct precursors of polyamines, is needed. Firstly, Arg is converted to ornithine or agmantine by either arginase or arginine decarboxilase (**Figure 1**). Arginase hydrolyses *L*-Arg into urea and *L*-ornithine (15).

There are two arginase isoenzymes (type I and II) which are encoded by different genes which differ in molecular and immunological properties and are located in different cell compartments. Type I arginase (termed as citosolic) is mainly located in liver and endothelial cells, while arginase II (mitochondrial) is produced in other tissues (16). Li et al. (15), transfected bovine endothelial cells with rat arginases I and II cDNAs and demonstrated a marked preference of these cells to produce urea and ornithine from arginine instead of nitric oxide (NO), which is synthesized by the nitric oxide synthase (NOS). This also applies to jejune (11). All of these observations point out to a cell-specific regulation of arginase and NOS action, which depends on the biological importance of either ornithine or NO to the cell (16).

In addition to the efficient competitive action of arginase over NOS, there is an alternative way to produce putrescine from arginine. In a first reaction, Arg is converted to agmantine by arginine decarboxilase (ADC) and then to putrescine by agmantinase (**Figure 1**). Although this mechanism has been

widely studied in plants, a similar mechanism in animals has been recently described but not completely elucidated (17,18). Agmantine has been detected in several tissues like small intestine and liver (17). It acts as inhibitor of NOS, ornithine decarboxilase (ODC) and polyamine transport, but it is an activator of *S*-adenosylmethionine decarboxylase (AdometDC), spermidine/spermine-*N1*-acetyltransferase (SSAT) and ODC-antizyme. Putrescine, spermidine and spermine from dietary sources are preferred substrates for transport than agmantine (19). The independence of ADC from exogenous stimuli other than intracellular concentration of arginine, confers to this pathway an special function on urea and polyamine formation. In fact, crucial enzymatic events on the urea cycle can be skipped by agmantine, through its conversion to *N*-acetylglutamate (NAG), which could be beneficial for certain urea cycle disorders (20). However, the complete participation of agmantine in metabolic stress is still unknown.

S-adenosylmethionine (Adomet) also known as SAMe, is the second important precursor substrate for polyamine production. This metabolite plays a central role in several cell reactions like transulfuration, methylation and aminopropylation. As a consequence of its transformation, other important metabolites like cysteine homocysteine, glutathione (GSH), neurotransmitters and polyamines are produced (21). SAMe supplied orally, is able to cross the intestinal wall and actively metabolized within it. However, its positively charged sulfonium ion restricts its passage from blood to peripheral cells (22). However, SAMe can cross the blood-brain barrier and produce neuro-transmitters which are important for treatment of depression. Also, the strict control of intracellular redox status via GSH production in mitotic competent intestinal cells, is important for decisions between proliferation or apoptosis.

Methionine adenosyltransferase (MAT) is the enzyme responsible to produce SAMe. The reaction involves first a rapid cleavage of the tripolyphosphate (PPPi) of ATP and transference of the adenosyl group to L-Met. The enzyme can be di- (MAT I), tetra- (MATIII) or heteromeric (MAT II), and possess phosphatase and transferase activities in two different active sites. While liver can exhibit the three isoforms upon development, in intestine and fetal tissues MAT III isoform is only found (23). Isoforms I and II are encoded by *MAT1A* gene, while isoform III is encoded by *MAT2A* gene and all three isoforms differ on substrate affinity (ATP and Met), feedback-regulation by SAMe, subunit rearrangements, ontogeny and suceptibility to NO and reactive oxygen species (ROS) (24). The lability to switch *MAT1A/MAT2A* gene expression in hepatic cells is needed for regeneration (22) and maintenance of the differentiated status of liver cells (25,26)

The control of SAMe on MAT gene expression is probably due to its transformation to 5-methylthioadenosine, a side-product in polyamine production (**Figure 1**). Also, the *S*-nitrosylation of MAT as consequence of NO presence can be avoided by GSH action suggesting that *in vivo* MAT exists in two forms in equilibrium (24). Due to the competition among NOS and arginase (and ADC), it seems likely that all of these orchestrated events are commonly connected to the same stimuli derived from intracellular concentration of SAMe and arginine.

The first reaction in polyamine biosynthesis is ornithine decarboxilation by the ornithine decarboxilase (ODC) to produce putrescine. ODC also activates DNA for synthesis and is essential for mitochondrial function (1). Mammalian ODC has one of the shortest half–lives described for mammalian proteins and is a homodimeric and unstable protein (2). ODC follows a circadian rhythm (27), responds to quality and quantity of dietary protein (28-29) and is regulated mainly at transcriptional level (29).

In a second reaction, *S*-adenosylmethionine is decarboxylated by AdometDC. Although the main action of SAMe is as methyl donor (85%), around 10% is used for polyamine biosynthesis. In contrast to the extensive studies on ODC activity regulation, AdometDC has been poorly explored, but the up regulation effect of SAMe and down regulation by its products (5'-MTA, SPd and SPm) on its gene expression and action is well established (30). Its over expression in *Xenopus* embryos lead to a programmed cell death of maternal cells (31). After overnight fasting, there is a simultaneous reduction of both ODC and AdometDC activities (29). Their activation depends on amino acid absorption velocity rather than *in situ* stimulation of local hormones in response to feeding.

ODC degradation is regulated by polyamines (Put<Spd<Spm) and is mediated by ODC-antizyme (OAZ). OAZ expression is produced by ribosomal frame shifting of the OAZ gene in response to the intracellular levels of polyamines. The active 23 kDa OAZ protein, binds to the 53 kDa-ODC monomer blocking the assembly of the active dimer. The ODC-OAZ complex is then targeted to degradation by 26S proteosome degradation but without ubiquitination. The ODC monomer is degraded by its carboxy- termini but not by OAZ (32). OAZ also prevents polyamine uptake into cells (33). Although the OAZ-ODC regulation mechanism has been extensively studied at structural and gene expression levels (34), many studies have reported certain ODC degradation independent of OAZ action (33-35). This is due to the regulatory action of the OAZ inhibitor (OAZI), which is an ODC-like protein without enzymatic activity that binds OAZ with higher affinity than ODC, reducing OAZ availability (32) leading an increase in ODC activity. Another possible explanation for the OAZ independent- ODC degradation is by the *S*-nitrosylation of the Cys^{360} residue of the active site of ODC (36). Spermidine and decarboxilated SAMe levels regulates ODC action by modifying activities of

AdometDC and MAT respectively (7,33). However, it is unlikely that this regulation is pre-translational since blocking synthesis of spermidine (SPd) or spermine (SPm) does not account for putrescine accumulation nor OAZ action on ODC. In fact, OAZ gene expression follows a sigmoidal behaviour from the early phase of cell cycle S to G_2/M, and its action over ODC is critical at early S (37).

Spermidine synthase (SPmS) or spermine synthase (SPdS) are not-rate limiting biosynthetic enzymes of the polyamine pathway. Although the gene expression and action are largely influenced by ODC and AdometDC activity, their action on sustaining SPd-to-SPm intracellular levels is more important. SPms and SPdS are directly inhibited by decarboxylated SAMe and 5´-MTA, and they are also more stable proteins than ODC and AdometDC. However there is an inducible nature of SPmS during cell proliferation, particularly in high cell density systems and in response to growth factors like TGF-β (38). On the other hand, SSAT is highly regulated and has also a rapid turnover rate like ODC and AdometDC.

Also, the combined effect of spermidine/spermine-N1-acertiltransferase (SSAT) and polyamine oxidase (PAO) constitutes an efficient retro-conversion route to control SPd and SPm intracellular levels. Their action leads to production of acethylated and oxidized by-products of SPd and SPm that can be excreted from the cell. While SPdS and SPmS show a more constant activity (as exerted by gene expression and low degradation of these enzymes), SSAT has an exceptional inducible behavior that do not encompasses those of the rate limiting enzymes, in fact it is complementary (while ODC, SPmS and AdometDC are TGF-β inducible, SSAT is not) (38). The SSAT gene is degraded by the ubiquitin-26S proteasome pathway (2) and its conditional over-expression is sufficient to reduce polyamine pool and arrest growth (39). A third participant of

polyamine degradation that is emerging is spermine oxidase (SpmO; **Figure 1**), however there are few reports about its action and regulation (40-41), thus it is necessary more studies about this participant to determine its exact role in polyamine homeostasis.

The production and creative team: Is there more than one director?

One of the most interesting facts of the polyamine pathway is the strict enzymatic control for maintaining the concentration ratio of polyamines. It is widely accepted that ODC, AdometDC and SSAT are the principal actors on this scene, so extensive studies on polyamine metabolism are focused on these three enzymes. Also, since ODC (anabolic) and SSAT (catabolic) obey to physical stimuli like osmotic pressure, it has been accepted that any cell detects polyamine depletion by simple concentration effect. However, so many questions arise from this fact like, why intracellular concentration ratio of Put/SPd/SPm in no proliferation stage is different among organs? or why under stressed conditions or growth stimulation some cells overproduce an specific polyamine while others do no. The answer underlies in both hormonal sensitivity and enzyme biosynthesis capacity of a specific organ besides the possible contribution that isoenzymes patterns of distribution in organs could contribute.

Hormonal stimulation and gene expression is a consequence of either systemic biodisponibility of substrates or specific signaling from intracellular origin. Hormonal activation of enzymes of the polyamine pathway resembles that of protein synthesis (**Table 1**). Amino acids can itself control the gene expression of the main substrate donor- enzymes (MAT and arginase) for the polyamine pathway. For instance, the biodisponibility of ornithine, Arg or Gln accounts for intracelullar activation of arginase and NOS, while methionine does

for MAT activity. If Arg is not degraded by arginase, it can be sequestered by NOS, resulting in MAT *S*-nitrosylation and methionine losses.

On the other hand, insulin stimulates all enzymes of the polyamine pathway and NOS, reduces arginase activity and does not have effect in MAT. Interestingly, while cortical steroids increase NOS (42), MAT and arginase activities, glucagon reduces NOS action (43) but it activates arginase and MAT. The simultaneous stimulation of all the polyamine enzymes is feedback regulated once metabolic demands for polyamines are fulfilled. ODC is also activated by histamine, cortisol, neuropetides, and in intestine by luminal amino acids like Gln, Arg and ornithine and IGF-1 (33). AdometDC has two upstream promoters sites for insulin, and responds to IGF-1, EGF TGF-β1 and AMPc (30). Last, SSAT, SPdS and SPmS are regulated pre- and post-transcriptionally by glucagon, GH and cortical steroids. Inhibition of ODC activity by α-difluoromethylornithine (DFMO), increase AdometDC but has no effect on SSAT activity.

Polyamine production and homeostasis are also regulated at transcription level. Firstly, Arg (44) and Met (23) mobilization for polyamine biosynthesis is probably regulated by the CCAAT enhancer binding protein (C/EBP). At low polyamine concentrations, ODC activation is mediated by its own gene expression by *c-myc* (2) This proto-oncogene also has target sites on MAT (23), SPdS and other related cell cycle genes (45).

ODC gene contain three potential binding sites for c-Myc and after its stimulation there is an increase in cell growth rate and S-phase entry is promoted in HeLa cells (46). Its regulation during normal cell proliferation is also mediated by a well defined c-Myc/Max/Mad network and when combined with retinoblastoma tumor supressor pRb, the cell transformation is regulated.

AdometDC is also regulated at transcriptional level (29) but the exact mechanism of up regulation is unknown (47).

On the other hand, the catabolic enzyme SSAT induction is regulated at gene transcription, mRNA translation, stabilization and protein degradation levels. Spd and SPms induce SSAT expression but depletion of putrescine represses it expression. Also, the absence of intracellular amino acids or just the precursor ones activates its expression. This combinatorial effect between amino acid sufficiency and their channeling to polyamine precursors can be explained by transcription factor recruitment. First, in presence of enough polyamines (mainly SPD and SPm), the nuclear factor erythroid (NF-E2) related factor (NRF2) binds to a *cis*-element located in the SSAT gene promoter which consensus sequence is TATGACTAA known as polyamine response element (PRE) (48,49). In murine embrionic cells, amino acid insufficiency can also up regulate gene expression of a polyamine modulated factor (50), which is almost identical to human polyamine-induced factor (PA-1). Dimerization of NRF-2 and PA-1 can also up regulate SSAT transcription either as a consequence of amino acid deficiency or by polyamine analogues (51). Beside these forms of SSAT gene expression, there are other transcription factors related to amino acid and energy mobilization that deserve special attention and will be discussed later.

Hearing the prompter: Polyamines, amino acids and signal transduction

Although there is a substantial knowledge about amino acids and other nitrogenous compounds as signaling molecules, studies with polyamines are scarce. Formerly, it was discussed that amino acids are involved in polyamine pathway activity and expression at different levels. Theoretically, mobilization of amino acids both for protein synthesis or for polyamine production are anabolic events, so initially there must be a growth stimulation signal mediated by insulin

or growth factors. Under these conditions, tyrosine kinase-like receptors are activated and they elicit stimulus principally under three ways (**Figure 2**).

The route mediated initially by the son-of-sevenless protein (SOS) to p90 activation is stimulated directly by intracelullar concentrations of polyamines, mainly putrescine and spermidine (52). The downstream effect of this activation results in a primary expression of three proto-oncogenes that alone (MYC) or in a dimerized form (JUN:FOS) are prerequisites for ODC induction (52). Spermidine itself positively stimulates the phosphorilation cascade at tyrosine kinase, ERK and *c-myc* levels. Also, putrescine stimulation results *c-fos*, *c-jun* activation. Additionally, although there are many ways to activate the phosphatidilinositol 3-kinase (PI3K), the phosphorilation of P13K by the oncogene Src leads to a downstream activation of c-myc and ODC gene transcription although a cross talk between the Ras-MAPK and p13K-mTOR pathways seems to be a requisite for polyamine increase and malignant transformation (53).

Amino acid-dependent stimulation of protein translation and polyamine pathway converges at the Akt-mTOR point (54). A downstream effect of the cascade by this route leads to the correct formation of the eIF4E-A-G complex by promoting the phosphorilation of 4E-BP1. Phosphorilation of 4E-BP1 is mediated by the mammalian target of rapamycin (mTOR also called FRAP or RAFT). In a by side reaction, mTOR also can promote $p70^{s6}$ phosphorilation that in turn phosphorilates S6 protein and stabilized the ribosomal complex to initiate translation. The primary evidences of mTOR-mediated regulation of polyamine biosynthesis is through transcriptional down-regulation of ODC gene (55). It has been proposed that mTOR enhances gene transcription of genes with long 5'-UTR like ODC and c-myc. However, it is possible an alternative pathway for c-myc stimulation and gene transcription of its target genes, since Akt mediates

sensitivity and consequences of mTOR inhibitors on cell proliferation (55). Also, the nutritient sensitivity of mTOR depends on its association with a protein called 'RAPTOR' which enhances mTOR actions over $p70^{S6k}$ and eIF4E (56), but its participation of c-myc transcription or regulation has not been reported yet.

It is noteworthy that initial activation of the three routes can be either stimulated by growth factors (tyrosine receptor) or amino acid (Akt-mTOR point) at initial steps but not backward. Polyamines instead, although in marginal concentrations within the cell, can elicit stimulation up (tyrosine kinase receptor) or down (nuclear proteins newly synthetized), and the polyamine gradient-like effect can be reproduce at almost any condition of the cell. In fact, spermidine concentration is even higher than the other two polyamines in not proliferating state (7), so signaling can be guaranteed. This feedback control has been recognized as the ODC-oncogene loop. Furthermore, the effect upon its own production, polyamines also participate in phosphorilation and/or stabilization of many proteins involved in structural morphogenesis which are particularly important for cell restitution in intestinal tissues (57-58). However, it seems likely that this interaction are not restricted to just one but to all three polyamines (19).

Changing the scenario: Metabolic readjustments in undernutrition.

Functional restoring of organs to malnutrition implies firstly the replenishment of energy stores and then cellular proliferation. Since there are specific hormonal arrangements (e.g. glucagon, insulin and IGF's) before and after nutritional recovery (59), there is a primary amino acid disposal for energy. Once energetic demands are fulfilled, amino acids can undergo other anabolic effects. Switching nutrient consumption from energy store replenishment to its usage for cell growth is also mediated by cell signalling (60) and gene transcription (61). Decreasing of available substrates comes with increases of

serum glucagon and cortical steroids due to stress and with a reduction on secretion and/or action of insulin, and growth factors.

In adittion, there is a marked fatty acid (FFA) mobilization and other substrates from adipose tissue to the liver (**Figure 3A**) and activation of specific transcription factors such as the peroxisome-proliferator activated receptors (PPAR's). The efficient programming during the energy replenishment period is mediated particularly by PPARα, since its gene contain a glucocorticoid response element (62), is sensitive to dietary factors (61), is widely distributed in most tissues (63) and can be efficiently activated by many endogenous FFA (64). Once protein is synthesized, and when hormonal status switch from catabolism (glucagon and glucocorticoid mediated) to anabolism (growth factor and insulin) there is a down-reduction on PPARα expression. In fact, this nuclear receptor follows a diurnal rhythm (62) but when it is not bound to its ligand is a labile target for the ubiquitin-proteasome system (65), coinciding with hormonal changing and energy substrates (carbohydrates instead of FFA) biodisponibility.

PPARα is also involved in amino acid and polyamine biosynthesis. Traditionally, c-myc has been recognized as a unique transcription factor that promote gene expression of many growth and DNA synthesis related proteins including ODC and SPdS as an example. Other like C/EBPα up-regulate gene expression of many enzymes of the urea cycle (44). Kernsten et al. (59), found a negative influence of activated-PPARα over most of the urea cycle enzymes including arginase and ornithine transcarboamylase (OCT; **Figure 3A**). Since it has been demonstrated an interaction between C/EBPα and PPARγ, the principal PPAR in this tissue (66), in the adipogenesis process, it is possible that PPARα can interact in the same way in liver and intestine to exert its inhibitory action. Another possible mechanism could be a trans-effector or trans-

repression (67) effect mediated by competition for CREB-binding protein (CBP) and p300 co-activators which are also recruited by PPAR's (67).

Kernsten et al. (59), also reported a negative action of PPARα on gene expression of SPdS and SSAT genes of the polyamine pathway. Both enzymes control the specific ratio of SPm-to-SPd within the cell, which in turn can serve as a 'latent' internal signal (52) to reactivate the polyamine metabolic pathway (**Figure 3B**), once the PPARα-mediated repression is switch off. In addition, activation of PPARα with Wy14,643 results in a dose independent action in c-myc gene expression but an increase in ODC action but activation with conjugated linoleic acid (CLA) resulted in up-regulation of ODC activity. Also up to now, only in the SSAT gene has been recognized two PPAR response elements (PPRE) in colon cancer cells (68). PPRE-2, at +48 bases relative to the transcription start site, is required for the induction of SSAT by ligand activated -PPARγ and PPRE-1, at–323 bases relative to the start site, is not required for the induction of SSAT by both PPARδ and PPARγ but is less responsive. These observations lead to the conclusion that PPARα can act on polyamine pathway as a trans-acting element as well as a target molecules for cell signaling. However, these theoretical inductions deserve future studies.

Concluding remarks

New tools in molecular nutrition have revealed many undercover aspects of polyamine metabolism. As participants of internal gossiping between substrates, like amino acids, polyamines have clarified some of the old questions about their action. Then, it should be thinkable that as far as we go with molecular tools, more specific activities of putrescine (Put), spermidine (Spd) and spermine (Spm) will be disentangled. For instance, from an structural point of view we can go forward in the near future on studying interactions with

other important biomolecules, since its role as triggering activators and signaling molecules may itself constitute a possible new important mechanism such as phosphorilation, acethylation and many other protein modification reactions.

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Table 1. Regulation of the polyamine pathway and related enzymes.*†

Group	MAT	Arginase	ODC	Adomet DC	SPdS	SPmS	SSAT	NOS
Hormonal Response								
Insulin	- or NA	-	+	+	+	+	+	+
Glucagon	+	+	-	-	-	-	-	-
Cortical Steroids	+	+	+	+	+	+	+	-/+
Growth Factors	+/-‡	+	+	+	+	+	+/-‡	+
NO/ROS Response	-	NA	+	NA	?	?	?	-
Transcriptional Regulation								
c-myc	+	NA	+	-	+	?	?	-
C/EBP	+(β)	+(β,α)	+	?	?	?	?	+(β)
PPAR§	NA	+/-	+	NA	-	?	+PPRE	NA

* MAT = Methionineadenosyltranferase, ODC= Ornithine decarboxylase, AdometDC= *S*-adenosylmethionine decarboxylase, SPdS = Spermidine synthase, SPmS= Spermine synthase, SSAT= Spermidine/Spermine-*N1*-acetyl transferase, NOS= Nitric oxide synthase, ROS= reactive oxigen species ; C/ EBPα,β = CAAT/Enhancer Binding Protein Alpha or Beta isoforms; PPRE = Peroxisome proliferator response element

† Symbols= (+) stimulation, (-) repression, (?) unknown, (NA) no action.

‡Particularly Hepatocyte growth factor (HGF) but not growth hormone in hepatic MAT; Growth Hormone (GH) but no Transforming growth factor β1 (TGF-β1) in liver SSAT.

§ Stimulation or repression either directly (existence of an upstream PPRE) or indirectly by other unknown mechanisms.

FIGURE LEGENDS

Figure 1. The Polyamine Pathway and by-side reactions. Abbreviations: Arginine decarboxylase (ADC), Ornithine decarboxilase (ODC), Ornithine transcarboamylase (OCT), S-adenosylmethionine decarboxylase (AdometDC), Spermidine synthase (SPdS), Spermine synthase (SPmS), Spermidine/Spermine-*N1*-acetyltransferase (SSAT), Polyamine oxidase (PAO), 5-´methylthioadenosine (5-´MTA), *S*-adenosylmethionine (SAM), Spermine Oxidase (SpmO).

Figure 2. Polyamine and Amino acids share common signaling pathways. Son-of-sevenless protein (SOS), phosphatidylinositol 3 kinase (PI3K), extracellular signal-regulated kinase (ERK), ERK kinases (MEKs), mTOR= Mammalian traget of rapamycin. See text for related other terms.

Figure 3. PPARα as a modulator of cell proliferation or stunting. Low nutrient availability (A), particularly amino acids lead to a favorable environment for PPARα which in turn stimulate down regulation of key enzymes of the polyamine pathway (B, grey boxes), favoring amino acid use as fuels.[59] Restoring energy during feeding reduces corticosteroid stimuli, ligand-activation by free fatty acids and PPARα protein degradation. For explanations see text.

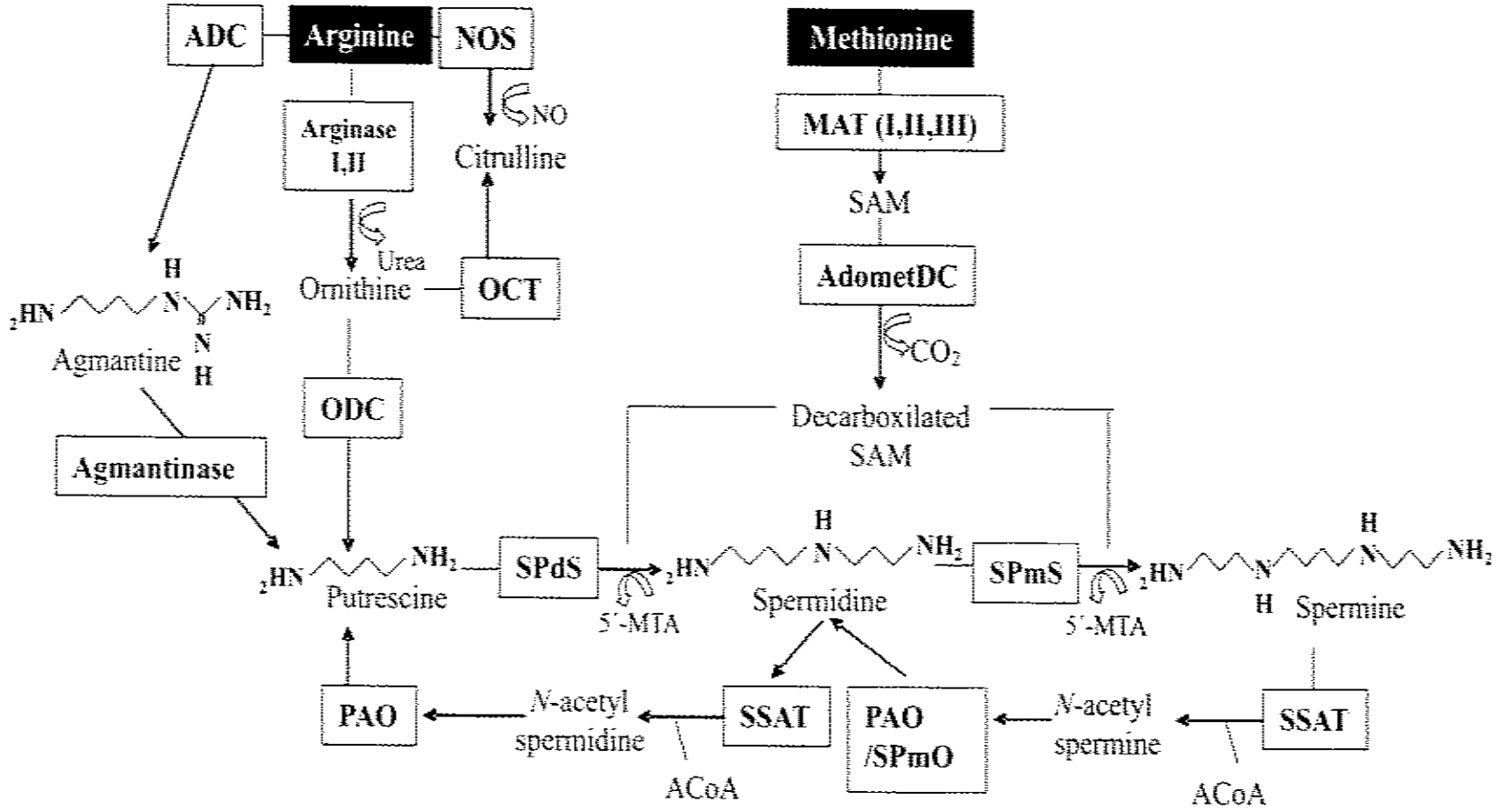
ADC
Arginine
NOS
Methionine
Arginase I,II
NO
Citrulline
MAT (I,II,III)
SAM
Urea
Ornithine
OCT
AdometDC
CO_2
Agmantine
ODC
Decarboxilated SAM
Agmantinase
Putrescine
SPdS
5'-MTA
Spermidine
SPmS
5'-MTA
Spermine
PAO
N-acetyl spermidine
SSAT
ACoA
PAO /SPmO
N-acetyl spermine
SSAT
ACoA

Figure 1

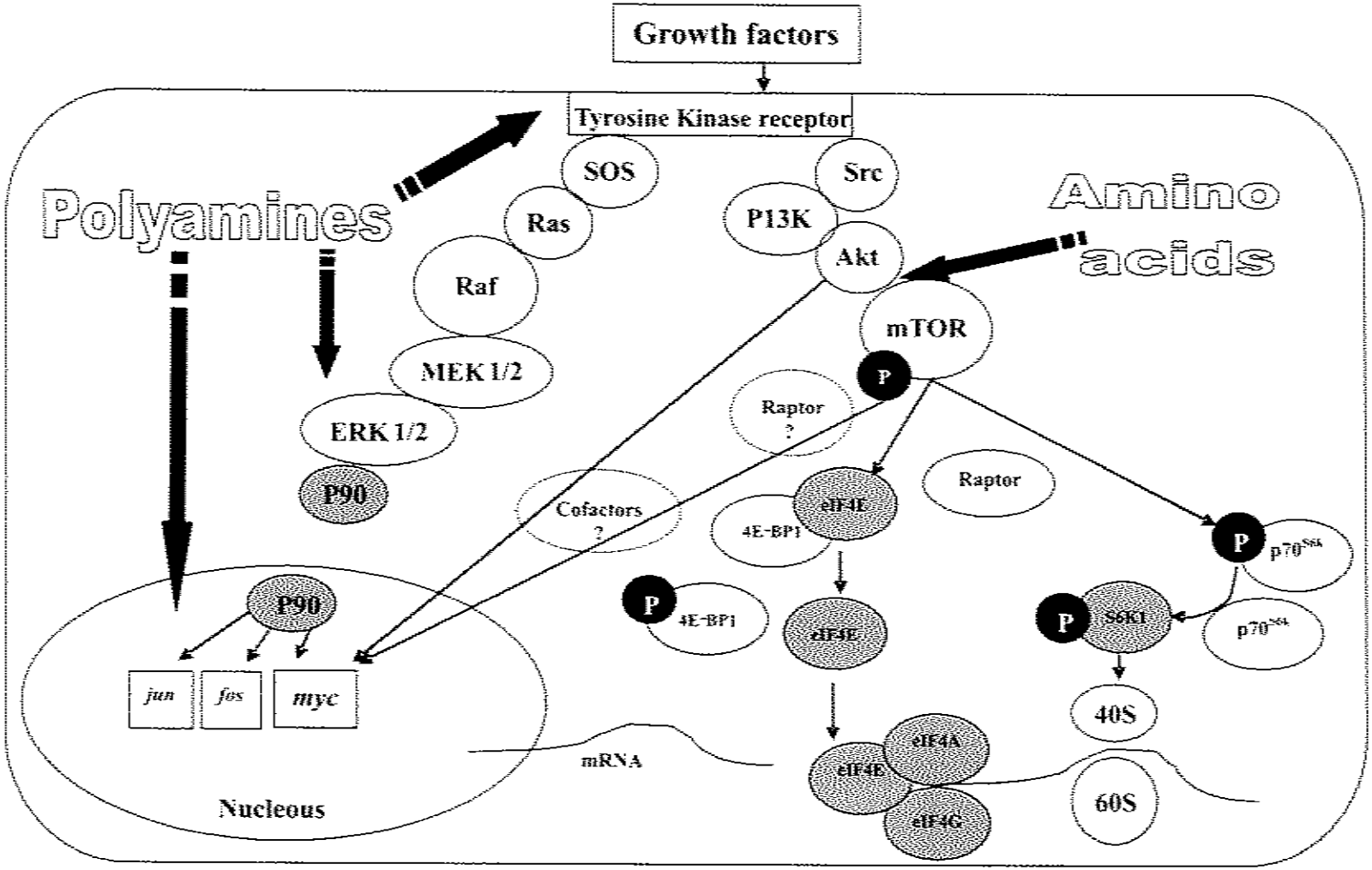
Growth factors
Tyrosine Kinase receptor
Polyamines
SOS
Ras
Raf
MEK 1/2
ERK 1/2
P90
Src
P13K
Akt
Amino acids
mTOR
P
Raptor ?
Cofactors ?
eIF4E
4E-BP1
Raptor
P
p70S6k
P90
P
4E-BP1
eIF4E
P
S6K1
p70S6k
jun
fos
myc
40S
mRNA
eIF4A
eIF4E
eIF4G
60S
Nucleous

Figure 2

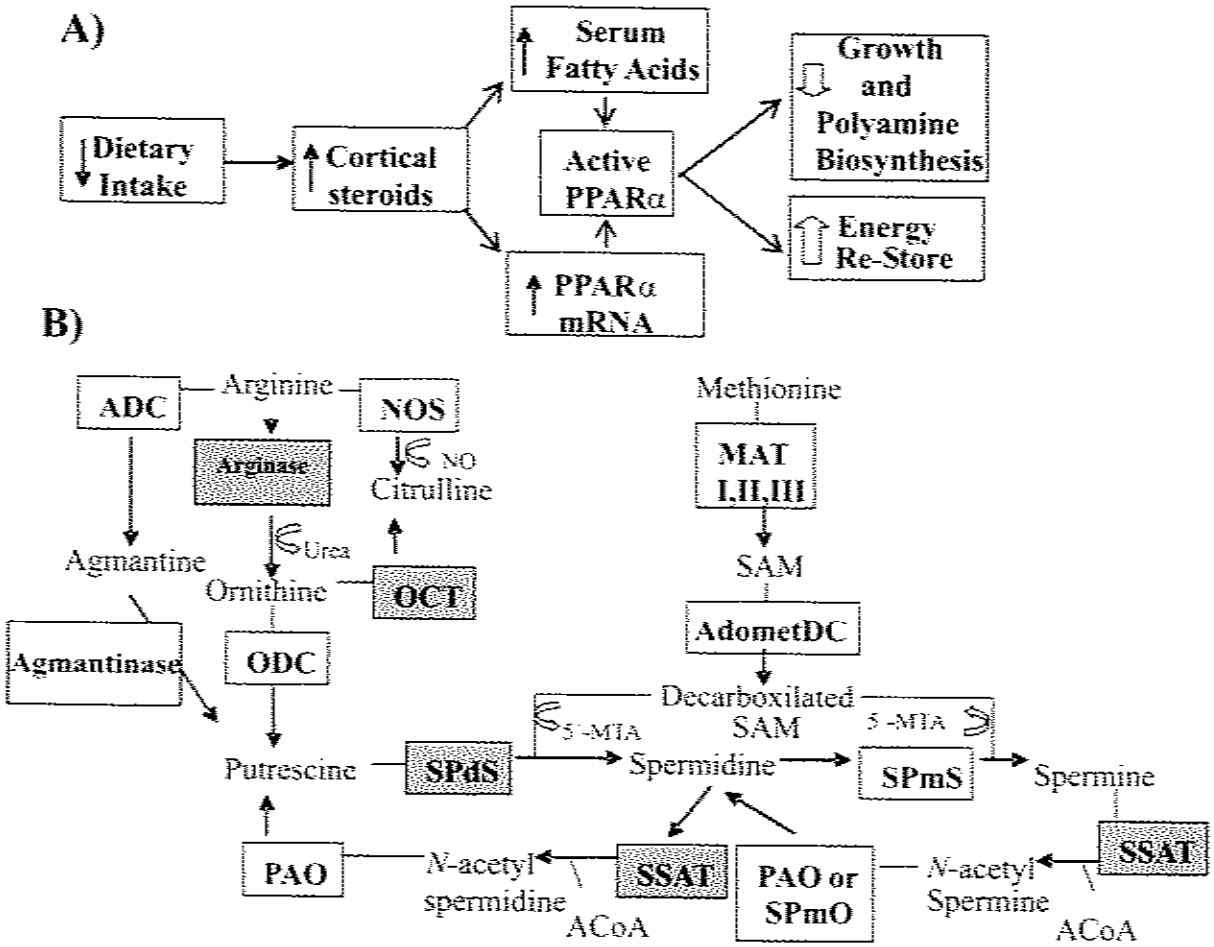
A)
Dietary Intake
Cortical steroids
Serum Fatty Acids
Active PPARα
PPARα mRNA
Growth and Polyamine Biosynthesis
Energy Re-Store
B)
ADC
Arginine
NOS
Arginase
NO
Citrulline
Urea
Agmantine
Ornithine
OCT
Agmantinase
ODC
Putrescine
SPdS
5'-MTA
Decarboxilated SAM
Spermidine
5'-MTA
SPmS
Spermine
Methionine
MAT I,II,III
SAM
AdometDC
PAO
N-acetyl spermidine
SSAT
ACoA
PAO or SPmO
N-acetyl Spermine
SSAT
ACoA

Figure 3

Nutritional improvement of enzymatically modified soy protein is more evident by feeding undernourished than healthy rats

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Re: Manuscript No. 04-030
Title: Nutritional improvement of enzymatically modified soy protein is more evident by feeding undernourished than healthy rats

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ABSTRACT

Background: Protein sources for special nutrition of infants are scarce and poorly evaluated. **Objective:** to evaluate in healthy and undernourished rats the nutritional quality of a modified soy protein as compared to two common protein sources used for infant nutrition. **Methods:** 16 healthy (Experiment 1) and 36 undernourished (Experiment 2) rats were fed with diets at 10% or 18% protein, respectively, based on: A modified soy protein fraction (MSP) with covalently bound Met to polypeptides of 1-10 kDa, a soy protein isolate + free Met (SPI) or casein (CAS). Protein quality indices and plasma urea were estimated in both experiments. Ileal and fecal amino acid (AA) digestibility, plasma AA and urinary NH_4^+ and urea were assayed in Experiment 1. Plasma proteins and free fatty acids (FFA) in Experiment 2 were measured before and during recovery. **Results:** Biological response of healthy rats fed the three diets was almost the same although CAS-fed rats excreted more urea and SPI more NH_4^+. Also, plasma Met and NEAA: EAA ratio were higher in MSP than in SPI fed rats. Protein intake, digestibility, plasma urea and FFA were comparable during the recovering period. MSP-fed undernourished rats used protein for growth and excreted more nitrogen in urine than CAS-fed rats during recovery. Higher serum proteins and lower urea level with the same nitrogen balance was found for MSP-fed undernourished rats, compared to the CAS-fed rats. **Conclusions:** These findings suggest that MSP could be a better option for nutritional recovering than SPI, and as good as CAS.

Key words: Modified soy protein, malnutrition, infant feeding.

INTRODUCTION

Modern soy-based infant formulas adequately support the growth and nutritional status of healthy infants (1). Soy flour- based formulas have been used for over 75 years for feeding children with allergy to cow's milk protein (CMP) or with lactose malabsorption (2,3). In a second generation of soy-based infant formulas 25 years ago, soy protein isolate (SPI) fortified with methionine (Met) replaced soy flour (4). Consequently, the American Academy of Pediatrics (5) recommended the SPI-based formulas as an alternative for breastfeeding or feeding with milk based- formulas when necessary.

In spite of its acceptable nutritive quality, SPI contains residual activity of trypsin inhibitors and allergenicity from its original source (6). SPI also contains phytochemicals which can be beneficial for adults but its impact on late infant sexual maturity remains questionable (2). Additionally, there is no proved nutritional benefit of SPI to sustain nutrition of malnourished children (7,8). In fact, infants with intestinal alterations derived from acute gastroenteritis can develop soy sensitive enteropathy upon its consumption (9).

Enzymatically modified soy protein by the plastein reaction followed by ultrafiltration, has been proposed as an alternative for special nutrition. It has been tested with successful results in animal models of hepatic cirrhosis (10) and the elderly (unpublished data). Furthermore, Kimura and Arai (11), evaluated two Met-enriched soy oligopeptides containing 10% protein, plus 1 and 3 % Met compared to the whole SPI, in healthy and malnourished rats. Met enrichment to the diet did not result in differences in healthy rats. However in malnourished rats, a higher protein efficiency of the modified oligopetide *vs.* the whole SPI was found at 1% Met, while at 3% Met, there were no differences. The conflicting results obtained by the authors for the undernourished group

were probably due to the low protein content in experimental diets, since 18% protein is recommended for nutritional rehabilitation of undernourished rats (12,13).

Recently, we developed a formula based on a technologically scalable Met-enriched soy hydrolysate fraction and found it better than SPI plus free Met for malnutrition recovery (14). However, our study just reported end point values at 21 days for different biological indicators during the recovery period. Also, the study did not explain differences in assimilation of the protein source in healthy and nutritional stressed conditions. Therefore, the aim of this study was to evaluate the nutritional quality of a similarly Met-enriched soy hydrolysate fraction in healthy and undernourished rats at different stages of nutritional recovering. The biological adaptation to a low protein diet (4%) during malnutrition induction is also discussed.

MATERIALS AND METHODS

Protein sources, reagents and chemicals. The enzymatically modified soy protein fraction (MSP) used in both feeding trials was previously described (14). MSP had a chemical score of 85 and 100 compared to the infant and pre-school children requirements (15) and a molecular mass distribution between 1 and 6 kDa. MSP composition was: 68% protein, 27% carbohydrate, 1% fat and 4% moisture. Soy protein isolate (SUPRO 500E, Protein Technologies Inc., St. Louis, MO., USA) with a protein and Met content of 90% and 1.2 g /100 g protein respectively, and ANRC-casein (Animal Nutrition Research Council) were used as reference protein sources. All other ingredients to formulate diets (vitamin and mineral mix, fiber, starch and sugar) were from Amersham Life Sci. (Little Chalfont, Buckinghamshire, UK). All other chemicals and reagents were of analytical grade and unless otherwise specified, they were from Sigma Chemicals Inc. (St. Louis, MO., USA).

Animals, diets and experimental procedures. Experimental animals were male & female weanling albino rats (*Rattus norvergicus*), 21 day of age, bred in at CIAD's colony. All experimental protocols were approved by the Institute's Ethical Committee. Rats were housed on individual Nalgene metabolic cages at 23°C with a 12 h light period (15) and feed and water were provided *Ad libitum*.

For *experiment 1* (Biological assay in healthy rats), 3 groups of 6 rats were fed with experimental diets based on the three protein sources mentioned above. Additionally, 7 rats were fed a protein-free diet to calculate metabolic losses. Diets were formulated at 10% protein as suggested by FAO/WHO (15). SPI diet was supplied with enough Met to reach the level in MSP. Chromium oxide (0.2% of the diet) was added as an indigestible marker for digestibility studies.

In *experiment 2* (Biological assay in undernourished rats), a Kwashiorkor-like malnutrition state was induced in 21-days old rats fed 4% casein-containing diet for 21 additional days. The 21 day period for malnutrition induction was based on either the metabolic response and histological findings from 12 rats fed the 4% casein diet and 6 control rats fed 18% casein diet, previously to the nutritional recovering period. Histological examinations were performed in hematoxilin-eosin (H&E) and Schiff's periodic acid (PAS) stained slides with 5µm jejunal and hepatic specimens. The nutritional recovery of 36 undernourished rats, randomly assigned to 3 groups (n = 12 each), was performed by feeding for 3 additional weeks with 18% protein-diets, based on the three protein ingredients mentioned above, composition in diets was recently reported (14). SPI and MSP had the same level of Met sufficient to cover requirements for the growing rat (16).

Protein quality and digestibility studies. Urine, feces and residual feed were collected daily and analyzed for nitrogen by the micro-Kjeldahl method (17). Urine was collected in acidic conditions (H^+) to avoid degradation of nitrogen compounds (Experiment 1). Protein quality was measured by the following indices: Nitrogen balance (NB), true (Experiment 1) or apparent (Experiment 2) digestibility (TD, AD), and protein efficiency ratio (Δ body weight/ g protein consumed). The protein digestibility-corrected amino acid scores based on either true digestibility (PDCAAS) or ileal nitrogen digestibility (PIDCAAS) were estimated in experiment 1 according to requirements of the pre-school infant (15).

Ileal and fecal digestibilities of individual amino acids (AA) and nitrogen (N) of healthy rats were performed according to Donkoh and Moughan (18). Rats were sacrified by cervical dislocation, and small intestinal sections, ileon and jejunum, were dissected. Intestinal sections were flushed with saline

solution, and digests were collected and dried. Dried pooled digests per section, feces and diets were analyzed for amino acids by HPLC (19) and chromium (digestibility marker) by standard atomic absorption techniques. Digestibility of each amino acid was calculated as follows: % digestibility: $[1-(Cr_f \times AA_d)/(AA_f \times Cr_d)]\times 100$, where Cr is the chromium content (ppm) in food ($_f$) and digest ($_d$) at jejune, ileum or feces and AA is the amino acid content (μM). The total nitrogen digestibility at ileum and feces was calculated based on the quantity and digestible proportion of each assayed amino acid-derived nitrogen.

Post-prandial plasma and urine metabolites. Blood was obtained by cardiac puncture of anesthetized rats and centrifuged at 1000 x g for 20 min. Determination of amino acids was carried out after precipitation of plasma proteins with 30 mg sulphosalicilic acid/mL plasma and centrifugation (2800 rpm, 15 min at 4 °C). Derivatization with OPA (o-ftalaldehyde) was done in 150 μL of supernatant + 19 μmoles of internal standard (α-aminobutiric acid) and immediately injected into the HPLC (19). Plasma and urinary urea were assayed with an urease-GLDH commercial kit (SPINREACT, S.A., Girona, España), plasma proteins (Randox Lab, Crumlin, Antrium, UK, Cat No. 2018) and free fatty acids by an enzymatic method (NEFAC, Wako Chemicals, Neuss, Germany). Urinary NH_4^+ was assayed by a microplate technique (20) at a 1:500 working dilution. Urinary urea and NH_4^+- derived nitrogen was expressed as percentage of the total nitrogen (% U_N) content in urine within the same day of analysis.

Statistical analysis. ANOVA tests were used to determine mean differences among the groups for all the variables studied, using protein intake as covariate. Differences were compared with the Tukey-Kramer test and were considered significant at $p<0.05$. Spearman rank correlation (r_s) was also used to study associations between variables (21).

RESULTS

Healthy rats.

Nutritional evaluation of healthy weaned rats fed with the modified soy protein (MSP), the soy protein isolate (SPI) and casein (CAS)-based diets is presented in **Table 1**. The total protein consumption of rats fed CAS diet was better ($p<0.05$) than those fed the SPI diet, but not different to those fed with the MSP diet. Rats fed CAS diet gained more weight at the end of the study than those fed with both soy diets. However, there were no substantial differences in the efficiency at which all healthy rats used protein for growth (Δ body weight/ g protein consumed) at the end of the study (**Figure 1A**). All groups presented the same nitrogen balance at the 10th day ($p>0.05$) but excreted different nitrogen compounds. Rats fed with the CAS diet excreted more ($p<0.05$) urea than those fed with soy diets while SPI- fed rats excreted more ammonia than CAS and MSP fed rats. CAS and MSP were equally digestible but better than SPI ($p<0.05$) and consequently PDCAAS and PIDCAAS values were better for CAS and MSP than for SPI. In general, nitrogen ileal digestibility (NID) was positively correlated with TD ($r_s=0.98$). Although the total NID (as calculated from individual amino acid digestibility– derived nitrogen) was the same for MSP and CAS (**Table 2**). There were some differences in individual amino acid digestibilities.

CAS had higher digestibility for Glx (Gln+Glu), Gly, Lys and branched chain amino acids (BCAA) than MSP, while higher digestibility for Thr, Arg, Ala and aromatic amino acids (AAA). Nine out of fifteen amino acids assayed were less digestible in SPI than MSP.

Total and individual concentration (μM) of amino acids after 2 h of feeding, were higher in rats fed CAS diet than those fed the soy diets (**Table 3**).

The ratio of non-essential to essential AA (NEAA:EAA) was lower in SPI fed rats (2.9) than those fed with CAS (3.5) and MSP (4.2) diets. There were several differences in amino acid responses between the two soy diets (Glu, Asn, Ala, Tyr, Trp, Met, Ile, Leu, Lys). Plasma Met in rats fed MSP was lower than CAS but higher than SPI. Therefore, in addition to the higher true and ileal digestibilities, rats fed the MSP diet presented also better ($p<0.05$) serum Met response than the group fed SPI.

Malnutrition induction.

Table 4 shows the biological adaptation of rats fed either a 4% or 18% casein diet. Initial body weights were 36 ± 5 and 38 ± 4 g, respectively. Rats fed the 4% casein diet did not lose weight during the study. In fact, these rats gained 23% of their initial body weight, but this value was 13 times lower than that of CAS 18% protein fed rats. Protein efficiency was increased in rats fed the 4% diet along the study while for rats fed the 18% diet, it increased exponentially by the first 8 days and decreased afterwards (**Figure 1B**). As shown in **Table 4**, fecal and urinary nitrogen looses were maintained by the rats fed the 4% protein diet while the 18% protein diet group, showed increasing looses.

Histological examinations (**Figure 2**) on CAS 4% rats showed intestinal hypoplasia, reduced villi height, enlarged crypts, reduced number of goblets cells and certain degree of tissue injury with mononuclear infiltration, as compared to control rats fed the 18% diet. The correspond effect upon hepatic tissue was characterized of a progressively reduced glycogen granules in the CAS 4% group, along the 21 days malnutrition induction period. Both tissues also presented low number of mitotic competent cells in CAS 4% as compared to CAS 18%rats

Additionally, although not statistically significant ($p>0.05$) the rats fed 4% diet tend to reduce progressively its digestibility. Both groups presented positive nitrogen balance throughout the study, but increases were only observed for the 18% group. Total plasma proteins (including albumin) in rats fed 18% protein diet were almost twice that of 4% protein fed rats at the end of the malnutrition induction period. There was a reduction in overall protein synthesis in rats fed 4% diet, as judged by the different level in total serum proteins and the albumin-to-total serum protein ratio (Alb/TP) and was the same in both groups. There was also, a tendency to reduce amino acid catabolism as judged by urea serum levels in 4% protein diet rats but was not significant ($p>0.05$). As expected for a malnutrition progression, free fatty acids increased ($p<0.05$) in rats fed the 4% protein level.

Recovery of undernourished rats.

Bioassay parameters and protein quality indexes obtained from rats under nutritional recovery are shown in **Table 5**. All the value comparisons are within the same stage of recovery and bioassay parameters are cumulative values. There were no differences in total energy, protein intake and free fatty acid serum levels ($p>0.05$), among the three groups in almost all stages of recovery. Nitrogen balance and apparent digestibility obtained with the three diets at any stage of recovery was the same, except for MSP at the 2nd week where N balance was lower and digestibility was higher than those for SPI and CAS. While faecal nitrogen excretion was the same at any stage of recovery independently of diets, urinary nitrogen excretion was higher in MSP fed rats than those fed the other diets, at any stage of recovery.

MSP was as good as CAS to sustain growth and better than SPI at the end of the study (**Table 5** and **Figure 1C**). Additionally, total serum proteins were increased in MSP-fed rats than those fed with CAS diet the last week of

recovery but this was not accompanied with increases in albumin serum levels (**Table 5**). Also there was a tendency of CAS and SPI fed rats to increase urea serum levels than those fed the MSP diet, although this was not statistical significant ($p>0.05$).

DISCUSSION

The environment of the intestine of neonates and children with metabolic disorders are a challenge for nutritionists (9,22). So, when the intention is to formulate a new protein-based product, it is advisable to evaluate it in a rat model which mimics the biological response of the target population. The MSP analysed here has several good characteristics for healthy and undernourished young infants: a) it has an acceptable chemical score for children and lactating infants (14), b) it has reduced anti-physiological and allergenic biomolecules as compared to SPI, according to previous studies in our lab, and c) the content of antiphysiological compounds after the ultrafiltration process is negligible. These characteristics are advantages in the preparation of formulations for young infants or people with malabsorption problems like the elderly. On the other hand, the presence of intact proteins with allergenic potential can be harmful for the ill small bowel in undernutrition (23).

Results in healthy rats fed the modified soy protein (MSP) do not account for a better protein quality than casein (CAS). Although the benefit of MSP consumption over SPI was found in these rats, MSP did not varied so much respect to CAS in term on nitrogen utilization, despite their differences in urinary urea excretion. There are conflicting data about differences in protein quality of soy and milk proteins in normal conditions. In animals, soy protein impact differently the whole body protein turnover (24) urea kinetics and inter-organ amino acid distribution (25) affecting its protein quality over casein. On the other hand, a higher digestion rate of soy protein contributes to an increase in amino acid deamination and liver protein synthesis in humans as compared with milk proteins (26). This phenomenon is accentuated with increasing levels of soy in the diet (27).

A possible explanation for differences in nutritional quality between soy and milk proteins, is their amino acid composition. The higher Arg: Lys content of soy proteins (≈ 1.2) as compared to that of casein (≈ 0.45) has been related to increases in serum glucagon levels (28). As a result, intense catabolism of amino acids can be expected since glucagon levels can promote gene expression of amino acid degrading enzymes in the liver (29,30). Here, MSP had an intermediate level (1.03) between CAS and SPI (0.43 and 1.22 respectively), so stimulation of amino acid degradation may be lower for MSP than SPI.

On the other hand, malnutrition triggers several metabolic adaptations that depend not only on the severity of malnutrition but also on the maturity stage of the animal (31,32). The protocol followed in this study intended to generate a protein deficient state in albino rats since protein energy malnutrition is still a public health problem in developing countries like ours (33). Animals in marginal nitrogen balance, with chronic intestinal and hepatic impairments and with decrease biosynthetic capacity were re-fed with three different diets of proven high quality (14). Protein efficiency resulted in an evident advantage of MSP over SPI but similar to CAS. However, the higher plasma protein and tendency to a lower urea production with the same nitrogen balance of animals fed MSP diet as compared to those fed CAS diet, are statements of a better metabolic response of undernourished rats to MSP. These findings correlate with the higher protein deposition in carcass previously found for MSP (14).

In healthy rats the total urinary nitrogen excretion for CAS or MSP diets was the same but higher than SPI and in undernourished groups it was higher with the MSP diet. Both observations can be explained by the fact that soy protein increases glomerular filtration in healthy or renal disfunctional dogs when

compared to casein (34). However, this issue did not impact sustantially the overall nitrogen balance nor the body protein deposition as previously reported (14).

The advantages of MSP over SPI were not evident in terms of digestibility since it was similar between groups in almost all stages during recovery. Although malnutrition is often accompanied of up-regulation of several genes for intestinal transporters and relaxation of the permeability to dietary molecules (35,36), normalization of the transport capacity for both soy ingredients was probably regained soon after re-feeding. Reeds and Garlick (37), reported an exponential fall in the daily rate of protein and methionine deposition from birth to 2 years old. While 1 g/kg/day of protein is deposited in the newborn, 1/4 is for the 0.5 year old child, and in both cases 3.5% of total amino acids deposited have to be of sulfur amino acids. The differential need in this important period of catch-up growth can be sustained with MSP-based diets since plastein reactions can be efficiently used to design ingredients with different amino acid quantities, without altering significatively their physicochemical and sensory characteristics nor the protein quality of the original source.

In conclusion, MSP based diets can be as efficient as casein to promote nutritional rehabilitation and growth promotion of children with malnutrition or with serious intestinal impairments. Enzymatic modification of soy protein can prevent the anti-nutritional activity from the original compounds and allergenic proteins present in SPI, that can alter systemic and local immune response of the undernourished small bowel. Also, the posibility of modelling MSP with a particular Met (or any other amino acid) content to sustain a correct methionine body depositon seem to be an advantage of MSP ingredients over free Met addition of soy protein isolates.

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Table 1. Bioassay and protein quality parameters in normal rats[1,2]

Parameters[3]	CAS (n = 6)	SPI (n = 6)	MSP (n = 6)
Initial weight	46.0±3.6[a]	42.3±1.5[a]	46.4±3.2[a]
Δ weight	46.1±3.6[a]	35.5±3.0[b]	36.5±4.6[b]
Protein Intake	11.7±0.4[a]	10.4±0.4[b]	11.1±.0.6[ab]
N Intake	1.9±0.1[a]	1.7±0.1[b]	1.8±0.1[ab]
Fecal N[4]	0.06±0.01[a]	0.10±0.01[b]	0.05±0.02[a]
Urinary N (U_N)[4]	0.40±0.07[a]	0.27±0.04[b]	0.46±0.06[a]
Urinary urea (%U_N)[5]	79.6±15.5[a]	50.9±11.2[b]	43.5±5.3[b]
Urinary ammonia (%U_N)[5]	0.02±0.02[a]	0.03±0.01[b]	0.01±0.01[a]
N Balance (mg N/24 h)[5]	140±10[a]	130±10[a]	130±10[a]
True Digestibility (%)	96.7±0.5[a]	93.9±0.5[b]	97.2±1.4[a]
PDCAAS[6]	97	94	97
PIDCAAS[7]	96	95	96
Serum Urea (mg/dL)	34.0±1.2[a]	29.0±1.2[b]	32.0±1.2[a]

[1] Values are expressed as medium ± SD. Cas= Casein, SPI+ Soy protein isolate, MSP= Modified soy protein.

[2] Different letter superscript between same line means significant differences, $p<0.05$

[3] Unless specified, reported values are in grams per 10 days.

[4] Value corrected by metabolic nitrogen losses by animals fed protein-free diet: fecal = 0.03, urine= 0.11 g/N.

[5] Percentage of urea or ammonia-derived nitrogen from total excreted nitrogen of three non-consecutive days

[6] Protein digestibility-corrected amino acid score (PDCAAS) based on FAO/WHO (1991) pattern for pre-school children and true digestibility value,

[7] Protein ileal digestibility-corrected amino acid score (PDICAAS) using apparent ileal nitrogen digestibility of each protein source for correction.

Table 2. Amino acid composition of protein sources and its apparent digestibility in normal rats.

Amino acid	Composition (g)[1]			Apparent Digestibility (%)[2,3]					
				Ileal			Fecal		
	CAS	SPI	MSP	CAS	SPI	MSP	CAS	SPI	MSP
Asx	7.4	11.5	11.4	96	95	96	96	94	95
Glu	19.9	20.9	18.9	98	96	96	97	95	96
Ser	5.2	4.5	5.3	94	96	94	96	95	95
His	2.5	1.8	2.6	95	97	95	97	95	96
Gli	1.6	4.7	5.3	97	98	95	96	97	93
Thr	3.1	3.7	4.8	96	91	97	98	92	97
Arg	3.0	7.3	6.4	95	94	98	96	90	97
Ala	2.8	5.4	4.8	85	94	96	96	94	98
Tyr	5.0	4.4	3.5	94	95	97	97	94	97
Met	3.9	5.1[4]	5.1	98	96	98	96	96	98
Val	4.8	5.0	5.0	96	97	94	96	95	96
Phe	4.6	4.4	3.5	94	96	96	97	95	94
Ile	3.9	4.8	5.8	95	98	93	99	97	98
Leu	8.4	8.0	8.2	98	94	95	97	92	96
Lys	6.9	6.0	6.2	97	93	95	95	96	97
Nitrogen	14.6	14.4	10.9	96	95	96	97	94	96

[1] Medium values of three replicates. Individual amino acid content is based on 100 g of protein (16g N) while nitrogen content is based on 100 g of dry matter. Cas= Casein, SPI = Soy protein isolate, MSP= Modified soy protein

[2] Values are expressed as percentage taken into account amino acid profile of diet and the individual amino acid content found in digests of a pooled sample from 6 animals per diet.

[3] Calculated from the nitrogen content and digestibility of each amino acid at ileum and terminal

[4] SPI Met content = 1.2 covalently bound + 3.9 free methionine.

Table 3. Post-prandial serum amino acid response of normal animals fed experimental diets[1,2]

AA (μM)	*CAS* (n = 6)	SPI (n = 6)	MSP (n = 6)	Pooled SEM[3]
Asp	14[a]	9[a]	12[a]	1.1
Glu	165[a]	112[b]	129[a,b]	9.2
Asn	41[a,b]	53[a]	27[b]	5.1
Ser	604[a]	394[b]	369[b]	11.1
Gln	453[a]	365[b]	388[b]	14.5
His	170[a]	151[a,b]	131[b]	5.6
Gly	461[a]	457[a]	452[a]	28.7
Thr	179[a]	64[b]	70[b]	5.9
Arg	119[a]	42[b]	47[b]	3.9
Ala	891[a]	729[b]	248[c]	27.4
Tyr	134[a]	146[a]	483[b]	15.8
Trp	83[a]	70[b]	33[c]	2.1
Met	86[a]	33[b]	55[c]	4.4
Val	138[a]	96[b]	85[b]	3.3
Phe	56[a]	65[a]	51[a]	4.4
Ile	102[a]	74[b]	56[c]	2.9
Leu	47[a]	46[a]	16[b]	0.8
Lys	1280[a]	590[b]	193[c]	26.6
Total	5094[a]	3870[b]	3140[b]	168.4
EAA	1916[a]	1131[b]	596[c]	67.4
NEAA: EAA[2]	3.5[a,b]	2.9[a]	4.2[b]	0.25

[1] Medium values of six animals per diet. Cas= Casein, SPI = Soy protein isolate, MSP= Modified soy protein.

[2] Non Essential amino acids (NEEAA)

[3] Medium Standard Error (SEM)

Table 4. Bioassay and protein quality parameters during induction of malnutrition[1,2]

Parameters[3]	1 Week		2 Week		3 Week	
	CAS4%	CAS18%	CAS4%	CAS18%	CAS4%	CAS18%
Δ weight	1.7±1.1^{a}	38.8±1.8^{b}	4.9±3.5^{ad}	83.6±5.8^{c}	8.4±2.3^{d}	112.7±9.3^{e}
Protein Intake	1.7±0.2^{a}	12.3±0.5^{b}	3.3±.0.4^{c}	28.8±.1.4^{d}	4.6±.0.6^{e}	44.2±.2.9^{f}
N Intake	0.3±0.0^{a}	2.0±0.1^{b}	0.5±0.1^{c}	4.6±0.2^{d}	0.7±0.1^{e}	7.1±0.5^{f}
Energy (Kcal)	153±17^{a}	267±11^{b}	303±34^{b}	627±30^{c}	417±57^{d}	961±63^{e}
Fecal N (F_N)	0.02±0.01^{a}	0.05±0.01^{b}	0.02±0.02^{a}	0.1±0.0^{c}	0.04±0.02^{a}	0.2±0.0^{d}
Urinary N (U_N)	0.03±0.01^{a}	0.7±0.1^{b}	0.02±0.02^{a}	1.3±0.2^{c}	0.04±0.03^{a}	1.9±0.4^{d}
N Balance (mg N/24h)	32±5^{a}	179±21^{b}	34±4^{a}	230±26^{c}	37±18^{a}	235±35^{c}
Digestibility (%)	94±3.2^{ab}	98±0.5^{b}	90±5.4^{a}	98±0.3^{b}	86±9.8^{a}	97±0.4^{b}
Plasma Proteins (mg/dL)	3.8±0.3^{ab}	--	4.2±0.6^{ab}	--	3.4±0.2^{a}	5.7±0.4^{b}
Albumin (mg/dL)	1.4±0.0^{ab}	--	1.7±0.2^{ab}	--	1.1±0.1^{a}	2.0±0.3^{b}
Alb/PT (%)	37.1±2.6^{a}	--	39.1±2.0^{a}	--	31.6±2.0^{b}	35±4.4^{ab}
Urea (mg/dL)	25.0±11.8^{ab}	--	14.2±3.5^{a}	--	15.4±2.5^{a}	30.0±4.6^{b}
FFA (μM)[4]	558±21^{a}	--	622±7^{b}	--	879±15^{c}	666±30^{b}

[1] Values are expressed as medium ± SD.Cas= Casein, SPI+ Soy protein isolate, MSP= Modified soy protein

[2] Different superscript letters within same line means significant differences, $p<0.05$

[3] Unless specified, reported values are expressed in grams per week as accumulated values

[4] FFA = Free fatty acids,

Table 5. Bioassay and protein quality parameters during malnutrition recovery [1,2]

Parameters[3]	1 Week			2 Week			3 Week		
	CAS	SPI	MSP	CAS	SPI	MSP	CAS	SPI	MSP
Δ weight	44.5±6.6^{a}	34.4±6.5^{b}	47.1±3.1^{a}	79.2±7.5^{a}	73.6±14.4^{a}	79.6±6.4^{a}	108±8.8^{a}	87.4±12.1^{b}	118±10.7^{a}
Prot. Intake	16.4±2.0^{a}	15.0±0.8^{a}	16.2±0.9^{a}	33.5±3.6^{a}	29.3±.2.1^{b}	33.9±.1.1^{a}	41.2±.5.9^{a}	36.7±.7.9^{a}	44.1±.0.9^{a}
N Intake	2.6±0.3^{a}	2.4±0.1^{a}	2.6±0.2^{a}	5.4±0.6^{a}	4.7±0.3^{b}	5.4±0.2^{a}	6.6±0.9^{a}	5.9±1.3^{a}	7.1±0.1^{a}
Energy	360±44^{a}	379±20^{a}	384±22^{a}	734±78^{a}	739±52^{a}	802±27^{b}	1085±143^{a}	1083±97^{a}	1248±37^{a}
Fecal N (F_N)	0.11±0.04^{a}	0.13±0.04^{a}	0.11±0.06^{a}	0.24±0.09^{a}	0.31±0.08^{a}	0.22±0.1^{a}	0.47±0.25^{a}	0.43±0.06^{a}	0.44±0.20^{a}
Urinary N (U_N)	0.61±0.24^{a}	0.62±0.3^{a}	0.91±0.28^{b}	1.7±0.5^{a}	1.5±0.6^{a}	2.6±0.4^{b}	2.9±1.1^{a}	2.8±0.9^{a}	4.3±0.4^{a}
N Balance (mg N/24h)	275±53^{a}	237±40^{a}	226±51^{a}	244±36^{a}	203±43^{a}	188±31^{b}	254±46^{a}	175±26^{a}	175±20^{a}
Digestibility (%)	95.8±1.7^{a}	94.7±1.5^{a}	95.7±2.4^{a}	90.6±2.1^{a}	93.4±1.6^{a}	95.9±1.6^{b}	92.7±2.2^{a}	93.7±1.1^{a}	94.8±2.4^{a}
Total proteins	5.3±0.7^{a}	5.2±0.1^{a}	4.8±0.5^{a}	5.0±0.6^{a}	5.3±0.6^{b}	5.3±0.7^{b}	6.4±1.2^{a}	5.5±0.4^{a}	5.7±0.3^{a}
Albumin	2.0±0.3^{a}	1.8±0.2^{a}	2.1±0.1^{a}	1.9±0.2^{a}	1.4±0.2^{b}	2.3±0.3^{c}	2.4±0.6^{a}	2.3±0.3^{a}	2.0±0.1^{a}
Alb/PT (%)	38.2±5.4^{ab}	34.0±3.3^{a}	44.4±2.9^{b}	39.3±5.7^{a}	27.1±4.1^{b}	44.0±1.6^{a}	38.6±13.4^{a}	41.2±3.1^{a}	35.3±1.3^{ab}
Urea	26.6±6.0^{a}	29.2±4.8^{a}	17.4±3.5^{b}	22.1±8.3^{a}	18.8±4.2^{a}	17.8±4.5^{a}	23.1±8.8^{a}	23.8±10.4^{a}	19.0±8.5^{a}
FFA (μM)[4]	663±67^{a}	603±117^{a}	673±211^{a}	789±91^{a}	674±116^{a}	791±54^{a}	765±92^{a}	738±36^{a}	733±43^{a}

[1] Values are expressed as medium ± SD. Cas= Casein, SPI+ Soy protein isolate, MSP= Modified soy protein

[2] Different superscript letters within same line and week of recovery means significant differences, $p<0.05$

[3] Unless specified, reported values for bioassay parameters are expressed in grams per week as accumulated values, except for plasma parameters. FFA = Free fatty acids; total proteins and urea are expressed in mg/dL; energy in Kcal

[4] Partial result were reported previously (14)

FIGURE LEGENDS

Figure 1. Dietary protein efficiency response under normal condition (A), during malnutrition induction (B) and nutritional recovery (C) of animals fed with experimental diets. Δw/ P Intake = Δ body weight (g)/ Protein Intake (g); casein (Cas), soy protein isolate (SPI) and modified soy protein (MSP).

Figure 2. Histological examinations in rat liver and jejune, before nutritional rehabilitation. Photographs L1 and L3 correspond to liver and J1 and J3 to jejune of rats fed with 4% casein diet. Photographs L2 and L4 correspond to liver and J2 and J4 to jejune of rats fed with 18% casein diet. Tissues were stained either with hematoxylin-eosin (100x magnification) or Schiff's periodic acid (200x magnification). M=mitoses, GC= Goblet cells, CR= Crypt, G= Glycogen.

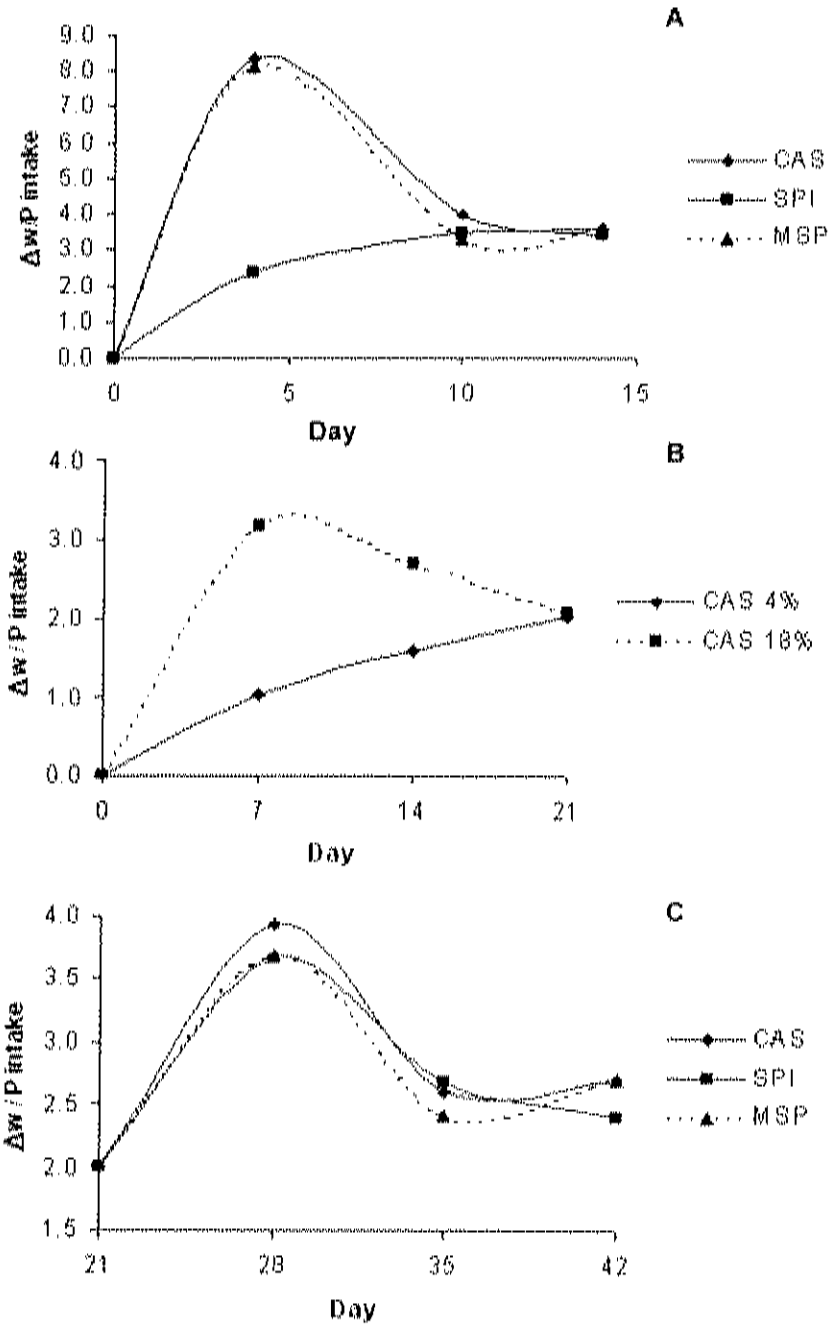
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Figure 1

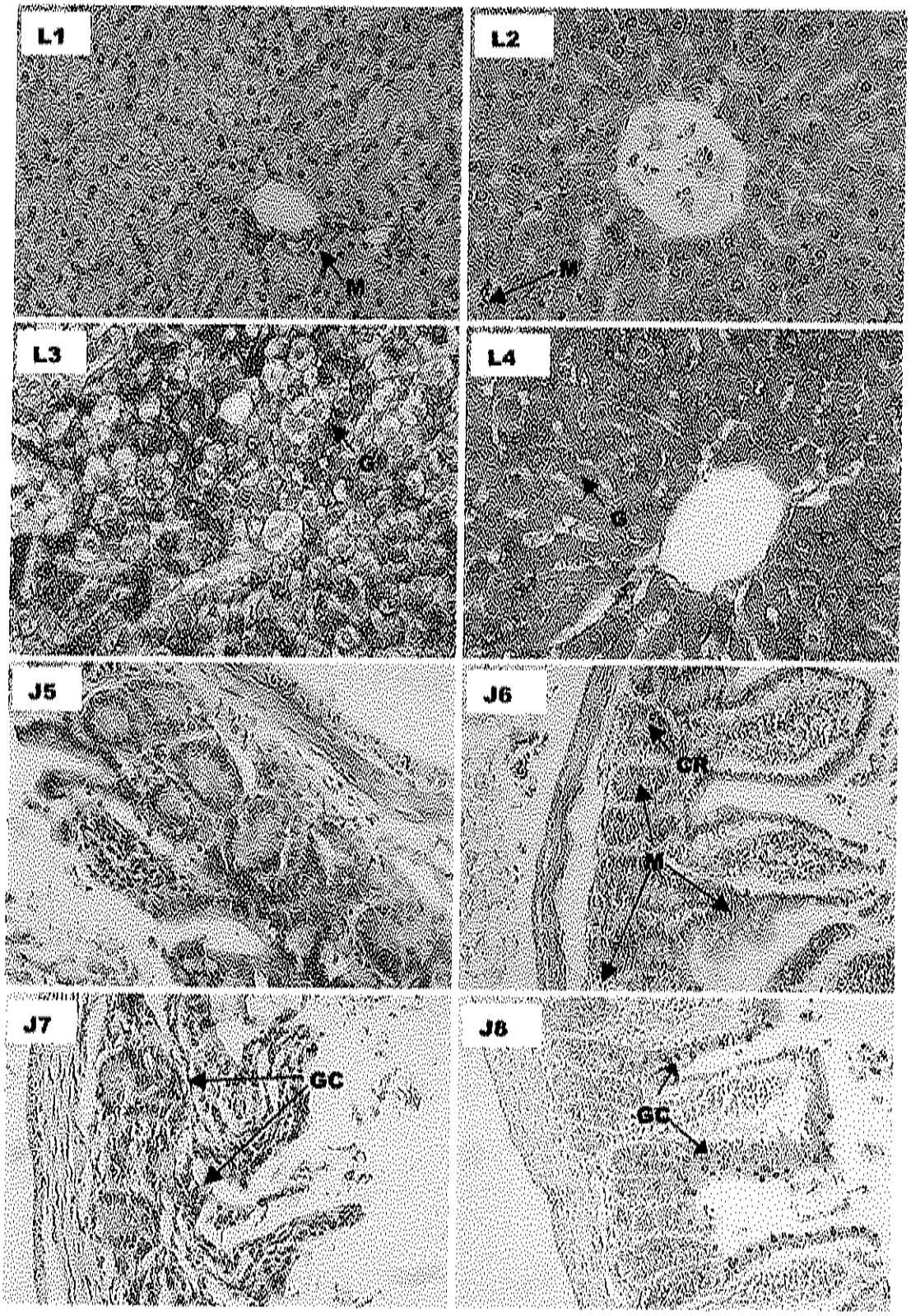

Figure 2

Polyamine metabolism in liver and intestine is regulated differently at transcriptional level by casein and modified soy protein in undernourished rats during recovery

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ABSTRACT

Functional recovery of organs after malnutrition requires polyamines which biosynthesis is influenced by dietary factors. The aim of this study was to evaluate the impact of oral administration of two highly nutritional protein sources on liver and intestinal functional recovery in undernourished rats. Twenty four undernourished rats (previously fed 4% casein diet for 21 days) were fed with 18% protein diets based on a methionine enriched soy protein hydrolysate (MSP18%) or casein (CAS18%). Bioassay parameters, plasma urea and hepatic, jejunal and ileal mRNA levels of enzymes and regulatory proteins of the polyamine pathway were evaluated before and after nutritional recovery. Immunodetection of $p53^+$ and $PCNA^+$ intestinal cells was also performed. Bioassay parameters were equal in MSP18%- and CAS18%-fed rats. However, Met and Arg intakes were higher while serum urea levels were lower in the MSP18% group than those of the CAS18% group. Undernourished rats had lower steady state mRNA levels of the polyamine enzymes and regulatory proteins ($p<0.05$) than control rats, except for a SSAT and OAZ in liver and OAZ in ileum. Until the 2^{nd} week of recovery, MSP18%-fed rats showed higher AdometDC mRNA level than the CAS18% group, on intestinal tissues. Reduced mitoses and $PCNA^+$ but equal number of $p53^+$ cells was found for CAS4%- rats compared to controls (CAS18%) or that observed in any recovered groups, independently of diet. Casein and MSP are both good protein sources for cell proliferation but differ in their capacity to enhance polyamine biosynthesis at transcriptional level during recovering of undernourished rats.

Keywords: Polyamines, undernutrition, soy, gene expression

INTRODUCTION

Polyamines are involved in DNA replication and cell proliferation (1). Their interaction with nucleic acids (2) is not restricted to normal cells but is also present in abnormal proliferation; therefore homeostasis must be strictly controlled (1). An orchestrated hormonal (3), neurochemical (4) and substrate bio-availability (5) combinatorial effect regulates participants of the polyamines' pathway (6). Tissues with high proliferation like liver are particularly susceptible to polyamine depletion in stress-like feed withdrawal (7) or folate deficiency (8). In intestine, polyamines are involved in cell migration and proliferation that result in the repair of mucosal lesions (9). Their intracellular depletion has been related to p53 gene expression (10), NF-κB activation (11) and growth arrest, but their sensitivity to apoptosis depends on the type of death stimulus (12). Also, migration of polyamine from depleted enterocytes up to the villi is decreased due to a reduced focal adhesion kinase and Ca^{2+}-Rhoa signaling (13).

Ornithine decarboxylase (ODC) and *S*-adenosyl methionine decarboxylase (AdometDC), the rate limiting enzymes of the polyamines pathway, respond to the quality of dietary protein (14) and are up regulated in stress but down regulated in starvation (7,15). The activation- of both enzymes is most likely to be more regulated by amino acid influx kinetics than by dietary-stimulated local hormones. Thus, intestinal activation of these two enzymes can be more efficient by using peptide-based diets than whole proteins, due to their differences in uptake and utilization (16). Several studies relate the benefits of peptides *vs.* whole proteins on intestinal and liver functionality in malnutrition (17,18) and small bowel resection (19). However, the effect of particular dietary protein on activity or gene expression of polyamine biosynthetic enzymes has not been studied.

On the other hand, Spermidine synthase (SPdS), Spermine synthase (SPmS;) and Spermidine/Spermine-*N1*-acetyl transferase (SSAT) are up regulated by glucagon and cortical esteroids. Additionally, high serum glucagon levels and mobilization of body energy stores are consequences of either malnutrition or ingestion of low quality proteins (20,21). Therefore, differences in gene expression of these enzymes can be expected in malnourished organisms. Also, polyamines, dietary protein and nutritional status modulate the stimulatory effect of Insulin-like growth factor-1 (22,23). The interaction among hormones and these three enzymes modulate the specific concentration of SPd and SPm and their proportions are important for deciding between cell cycle progression or apoptosis (24).

In this study, it was evaluated the mRNA levels of some enzymes and regulatory proteins involved in polyamine metabolism upon consumption of modified hydrolysate or whole high quality proteins in undernourished rats. The impact of these diets on the functional and morphological recovery of the intestine and in cell decision for apoptosis or growth was also examined.

MATERIALS AND METHODS

Animals and experimental diets. To evaluate the effect of modified soy protein (MSP) and casein administration on polyamine pathway gene expression in liver and intestine, 21 days old undernourished Wistar rats were used. A preliminary assay was performed to set the undernourished period at which macro- and microscopic signs of undernutrition were shown. All animals were supplied by the local inbreed colony of our institute. Both experiments were performed after approval of an internal ethical committee.

Firstly, sixteen rats weighing 36-38 g were housed individually in metabolic cages under controlled temperature and a 12 h light/dark cycle. They had free access to water and food. Twelve rats were fed a 4% casein diet (CAS4%) or 18% casein diet (CAS18%) for 21 days. Four rats of the 4% group were killed on days 7, 14 and 21 days and their malnutrition signs were registered. The proximal composition of diets was recently published (25). Twenty one days feeding were sufficient to ensure the metabolic disturbance of the organs to be tested (intestine and liver). Food consumption and body weight were recorded every third day to estimate average values. At the end of the treatment, rats were weighed and then anesthetized with an intraperitoneal injection of 6.25 mg/kg of body weight of tiletamine-HCl and zolazepam-HCl.

Nutritional rehabilitation with experimental diets was evaluated in twenty four rats weighing 51-54 g. As mentioned above, all rats were fed for 21 days with the CAS4% diet to induce a protein-energy malnutrition condition. After this period, two groups of twelve rats were recovered with diets containing either 18% casein (CAS) or modified soy protein (MSP18%). MSP is a polypeptide fraction (average mass = 1 to 10 kDa), produced by enzymatic hydrolysis and Met enriched by a plastein reaction (25). According to that study, MSP is highly

digestible in normal and undernourished rats. At days 7, 14 and 21 during recovery, 4 rats of each feeding group were randomly selected, weighed and anesthetized as mentioned above. Proximal composition of the 18% and 4% diets has been previously reported (25).

Biological samples. All anesthetized rats underwent the same surgical procedures. First, as soon as the abdominal cavity was opened, blood was collected in tubes with EDTA-K^+ by intracardiac puncture. The intestine was rapidly excised from the ligament of Treitz to the ileocecal valve and rinsed with saline to eliminate excess of blood. Ileal and jejunal segments were identified 2 and 30 cm far from the ileocecal valve. Three portions of 1 cm from either segment were cut. Two were placed in RNAse free sterile plastic tubes and frozen under liquid nitrogen and the other was fixed in formaldehyde 30% and embedded in paraffin for histological studies. These procedures were also applied to 300 mg portions of liver. Plasma was separated from whole blood by centrifugation at 1,000 x *g* and urea quantified with a urease-GLDH commercial kit (SPINREACT, S.A., Girona, España). Immediately after blood collection and organ removal, animals were killed by cervical dislocation. All biological samples were kept at –70°C until analyses.

Isolation of liver and intestinal RNA. Total RNA was prepared from individual frozen tissues using Trizol™ reagent (GIBCO BRL, Burlington, Canada) and 100 and 200 mg of liver and intestine, respectively, according to manufacturer instructions. The quality and quantity of RNA (suspended in DEPC treated water) was evaluated spectrophotometrically at 260 and 280 nm. RNA Integrity was confirmed using a 1% agarose-formaldehyde gel electrophoresis to analyze 5 µg of denatured total RNA, stained with ethidium bromide (26) and visual observation of ribosomal RNA bands under UV. After quality and integrity

confirmation, 4 RNA samples (20 µg each) were pooled per tissue (ileum, jejune and liver) for analyses.

Primers. Forward and reverse primers were designed from reported DNA sequences (GenBank) and are shown in **Table 1**. These were: Glyceraldehyde 3-phosphate dehidrogenase (GAPDH), Ornithine decarboxilase (ODC), *S*-adenosyl-methionine decarboxilase (AdometDC), Spermidine synthase (SPdS), Spermine synthase (SPmS), Spermidine/Spermine-*N*1-acetyltransferase (SSAT), ODC-antizyme (OAZ), Antyzime inhibitor (OAZI). All DNA sequences used for primer design were for *Rattus norvergicus* except for SPdS and SSAT. Since there were no sequences available for SPmS and SSAT genes at the time of design, conserved sequences of the homologous genes from human and mouse were used instead. Also, experimental protocol implies amplifications of both target and constitutive gene (GAPDH) at equal conditions, thus a Tm ≈ 60°C for all primers was conditionally set.

Semiquantitative RT-PCR. cDNA was obtained from 15 µg of each pooled liver or intestinal RNA-sample using Superscript™ first-strand synthesis system (Invitrogen, Carlsbad, CA) following manufacturer instructions, in a total volume of 60 µL. Controls for template contamination (RT-) and control RNA (RT+) were included. PCR amplifications were carried out using specific set of primers (**Table 1**) in a total volume of 50 µL as follows: 6 µL of cDNA (1.5 µg from total RNA), 30.5 µL water, 5.6 µL $MgCl_2$ (25mM), 4.2 µL PCR buffer 10X, (50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.001% gelatin), 1.4 µL dNTP mix (dATP, dTTP, dGTP, dCTP, 10 mM each), 2µL primer mix (20 µM Forward: 20 µM Reverse), and 2.5 U *Taq* Polymerase (SIGMA, St. Louis MI). GAPDH, ODC and AdometDC individual cDNA amplification were used to optimize the conditions and applied to all other genes, since higher levels of these three genes were expected. The conditions for linear PCR amplification of gene products were

determined by carrying out amplifications at two different cDNA concentrations (from 1.5 and 3 µg of total RNA). The amplification conditions were as follows: pre-PCR = 85°C/30 sec-94°C/3 min, PCR (35 cycles) = 94°C/30 sec-60°C/30 sec- 68°C/3° sec. All PCR products were then separated by electrophoresis on a 2% (w/v)-agarose gel and stained with ethidium bromide. Gel image was obtained and analyzed using Digital Science ID software (Kodak, Rochester NY) and the total band net intensity evaluated by densitometry. Relative concentration (pixels) of target gene versus GAPDH was then calculated for each set of transcripts and treatments.

Histology and immuno-histochemistry. Sections of jejune and ileum, were excised from 4 undernourished (4 rats fed CAS 4%) and control (4 rats fed CAS 18%) groups, before the nutritional rehabilitation period. Also, tissues from 4 rats fed CAS or MSP at 18% from 1st, 2nd and 3rd week of recovery, were analyzed. Tissues were immediately fixed in 3.7% formaline, dehydrated with alcohol and embedded in paraffin. Individual de paraffinized and rehydrated specimens were then stainned with Hematoxiline-Eosin (HE) by standard procedures. The number of mitoses found in 100 crypts (cumulative values from 4 rats) was then counted.

Immunodetection of p53 and proliferating cell nuclear antigen (PCNA) was performed as follows: Two re-hydrated tissues were peroxidase- inactivated with H_2O_2 at 3% for 5 min and then blocked with bovine serum albumin 1% (BSA). After washing, tissues were incubated with either a monoclonal mouse anti-PCNA (Clone PC10; DAKO, Denmark) or polyclonal rabbit anti-p53 phosphospecific (pSer^{392}, Biosource International Inc., Camarillo CA, U.S.A.) antibodies for 45 min at 1:1000 working dilution. After this, tissues were washed out (PBS plus 0.1%, Tween 20) and incubated with peroxidase-conjugated goat anti-mouse (DAKO) or swine anti-rabbit IgG (Biosource) respectively for 30 min

at a 1:2000 working serum dilution. After PBS washing, development was carried out by using diamino bencidine (DAB) and H_2O_2. $PCNA^+$ and $p53^+$ cells found in 100 crypts were counted and reported as "positive cells". Since the presence of both proteins means progression (PCNA) or arrest (p53) of the cell cycle, the ratio p53/PCNA (termed here as "growth arrest index" or "GAI") for specific tissues was calculated.

Statistics. Values were expressed as means ± SE. Comparisons between means were done by analysis of variance (ANOVA) and Tukey Kramer comparison test. All statistical analyses were considered to be significant at the $P < 0.05$ level. Spearman correlation rank test (r_s) was also used to evaluate mRNA steady state level patterns during nutritional recovery (27).

RESULTS

Bioassay parameters.

Rats fed the CAS4% diet showed significant lower weight gain compared to those fed CAS18%; consequently urea production on the former group was lower than the latter (**Table 2**). During recovery, all rats accepted both diets to the same extent, thus protein, energy and Glx (Glu+Gln) intakes were very similar. However, there was a tendency to increase energy consumption for rats fed MSP18% and therefore to gain weight at the third week, although the net use of protein for growth and maintenance was equal for both diets at any stage of recovery (data not shown)

Intestinal gene expression

Tables 3 and **4** show the differential mRNA levels in jejune and ileum of enzymes of biosynthesis (ODC, AdometDC, SPdS, SPmS), catabolism (SSAT) and regulatory proteins (OAZ, OAZI) of polyamines, before and during nutritional rehabilitation. Values of mRNA levels were normalized respect those of the GAPDH gene. Undernourished rats (CAS4% group) showed down regulation pattern for almost all enzymes and regulatory proteins in both intestinal tissues, except for ileal SPmS and OAZ, as compared to the control group (CAS18%-fed rats).

In jejune, ODC transcripts down regulation seemed to occur concomitantly with OAZ, OAZI and SSAT. Moreover, almost all the transcripts in ileum except ODC and AdometDC, showed a peak response after the 1st week of rehabilitation with MSP18%, and some of them had a second peak after the 3rd week. The mRNA levels of the regulatory proteins (OAZ and OAZI) were down regulated in both intestinal tissues during nutritional rehabilitation of MSP18%-fed rats, while those of the CAS18% group behaved erratically.

Polyamine gene expression in liver.

A down regulation appears to occur in all biosynthetic enzymes, up-regulation of catabolic enzymes and no effect on regulatory proteins mRNA steady state levels during induction of malnutrition due to the low protein intake (CAS4%) compared to CAS18%-fed rats (**Table 5**). Rats fed the MSP18% diet during recovery showed a progressive up-regulation of ODC, AdometDC and SSAT mRNA levels. SPdS and SPmS mRNAs behaved erratically during recovery in both groups. Except in 2nd week, fluctuations on OAZ mRNA levels behaved as those for ODC in MSP18%-fed rats but its inhibitory protein (OAZI) was only detectable after the 2nd week of recovery. In MSP18%-fed rats ODC gene expression correlated with Arg intake (r_s=0.84) and AdometDC with Met intake (r_s=0.99), while SSAT gene expression correlated with consumption of both amino acids (r_s>93).

Intestinal histology and immunohistochemistry.

At the end of undernutrition period, rats fed CAS 4% diet showed a significant reduction on villous height, crypt depth and total mitoses in 100 crypts, than their CAS18% counterparts. Additionally, there were more PCNA$^+$ cells in the 100 crypts counted from the CAS18% (96 cells, **Figure 1A**) as compared to the CAS4% (66 cells, **Figure 1B**) but p53$^+$ crypt cells were similar in both groups (38 *vs.* 35 cells, respectively). The growth arrest index (GAI = p53$^+$/PCNA$^+$) in CAS4% group was 1.3 times higher than that of the CAS18% group. However, the most evident difference was the pattern and distribution of p53$^+$ cells at villi top which was more evident in CAS18%- than in CAS4%-fed rats (**Figure 1C** and **1D**). During nutritional rehabilitation, neither CAS18% nor MSP18% showed significant differences in villus height, crypt depth or mitoses per crypt. However, GAI was 0.22 for the MSP18%-fed group and 0.30 for the CAS18% group due to a slight difference in p53$^+$ crypt cells (19 and 25 positive cells, respectively).

DISCUSSION

Protein-energy malnutrition (PEM) is one of the main causes of childhood illness in developing countries (28). PEM development depends on starvation time, energy and protein intakes and nutrients quality. Specifically, neurochemical (29) and hormonal (22,30) responses are consequences of protein quality and quantity. Therefore, alterations in cell signaling (31), gene expression (32) and cell growth (33) could be expected. These features accentuate the negative nitrogen balance state found in children with PEM (34). So, the development of new protein based products to treat children PEM is continuously needed (35) and their safer use must be supported by experimental evidences in animals, including those at molecular level.

Disturbances in small intestine are well known characteristics of critical food deprivation (36). Particularly, fasting or malnutrition alters cell proliferation, differentiation (7,37) and structure (38) of the intestinal mucosa. Here, feeding young rats with a low protein diet (CAS4%) for 21 days resulted in morpho- and physiological disturbances in ileum and jejune, similar to acute starvation. Same disturbances have been shown after acute fasting in young chicks (37), undernourished rats (39) and piglets (40). The loss of intestinal architecture in our animals was related to a reduced cell proliferation rate in crypts, as supported by the low number of mitoses and $PCNA^+$ cells (37,39).

Polyamine depletion in intestinal cells is also related to growth arrest mediated by p53 (10,11). The relation between tissue reactivity for this onco-protein and PCNA has been widely used to study the progression of different types of cancer (41, 42). In our case, a complete picture of how the cell accommodates to incoming nutrients for growth was obtained, by using both cell growth markers. Both, ileum and jejune from CAS4%-fed rats showed lower

PCNA$^+$ cells in crypts, than controls (CAS18%). Since both groups showed the same level of p53$^+$ crypt cells, the resulting growth arrest index (GAI) was higher than those animals fed the CAS4% diet. Growth arrest in these rats was also related to the down-regulation of mostly all enzymes and regulatory proteins of the polyamine pathway, as compared to controls (CAS18%).

During nutritional rehabilitation, the beginning of mucosal mass recovering occurred at the 1st week, independently of feeding diet. The healing of intestinal mucosa and its rapid cell restitution (9) is undoubtedly due to ODC and AdometDC action. This is supported by the fact, that ODC mRNA levels had an early peak at the first week which coincided with increments in PCNA$^+$ cells at crypt. These effects were normalised at the second week. A similar effect in both ODC gene expression and the rapid cellular restitution has been found in rats after small bowel resection (43).

On the other hand, soy protein consumption modifies cell growth at transcriptional level (44), as compared to casein. The inhibitory effect on cancer progression has been related to its high phytoestrogen- and low methionine (Met) content (45,46). Wang and Higuchi (47), demonstrated that free supplementation of Met (3 g/ kg diet) to a 200 g/kg soy protein isolate (SPI)-based diet did not increase intestinal polyamine levels in comparison with a not Met-supplemented SPI diet (47). In the same study, both SPI diets were lower producers of polyamines than a casein diet. Also, anti-physiological content of soy protein, limited its protein digestibility, having consequences that can resemble those of low quality proteins (32). Therefore, although the low production of polyamines derived from soy consumption is good for nutritional assistance of people with gastrointestinal cancers, is not an alternative for treating metabolic disorders in which cell growth or renewal is needed (37).

Modified soy protein obtained by enzymatic hydrolysis and synthesis with covalent binding of Met, has been proposed as an alternative treatment of under-nutrition (25). The Met-enriched soy protein hydrolysate (MSP18%) used in this study had a tendency to up-regulate polyamine biosynthesis at transcriptional level. Except for the 1st week of recovery, AdometDC mRNA level was higher in MSP than CAS-fed rats, while that of ODC fluctuated randomly throughout recovery in ileum and jejune. At least in jejune, it is possible that ODC activity was efficiently controlled by OAZ induction, as judged by the same pattern of reduction of mRNA levels of both during recovery. This phenomenon has been found during nutritional rehabilitation of rats subjected to small bowel resection (43). The same activation pattern of OAZ was found for OAZI in jejune of MSP18%-fed rats.

Thus, the modulation of ODC activity and expression during nutritional recovery, seemed to be orchestrated by ODC:OAZ and OAZ:OAZI complexes in a tissue-specific manner. OAZ is constitutively expressed in various rat tissues, is not regulated by intracellular putrescine content (ODC's product) and protects the cell against excessive polyamine accumulation (48). The ontogeny of both ODC and OAZ and their nuclear translocation may also contribute to ODC activity modulation (49). On the other hand, it has been demonstrated that MSP is highly digestible for undernourished rats promoting a better body nitrogen deposition than casein (25). Therefore, the efficient control of ODC:AOZ:AOZI mRNA ratios in MSP-fed rats can be a consequence of the rate of protein absorption. However, to our knowledge, there is no report about OAZI ontogeny or stimulation by diet, thus it is necessary to prove if there is real "triad affect" between these three participants of the polyamines' pathway in health and disease with more detailed studies.

ODC and AdometDC enzymatic activities are encompassed by their corresponding gene transcription (43) and respond to dietary protein (14,15). Thus, they are useful indicators in molecular nutrition. However, at least for ODC there is a circadian rhythm (4) and as assayed in starved turkeys, readably activity fluctuations are within the following 48 h after refeeding (7). In this study, during nutritional rehabilitation food restricted meals (half of normal consumption), were provided overnight and at the morning food was supplied *ad libitum* for those animals to be sacrificed 2 hours later. So, metabolic induction of any gene analyzed, including ODC, was more dependent on nutrient delivery and transcriptionally competent cells than to hormonal or SNC-stimuli.

Interestingly, up-regulation of ODC and AdometDC genes was likely to be dependent on amino acid delivery (15). This observation relies on the following facts: A) Total protein and energy consumption of MSP- and CAS-fed rats were the same throughout recovery, so a difference in energetic substrate bio-availability (especially lipids) that may enhance polyamine biosynthesis (47) was not an issue. B) Hepatic ODC and AdometDC gene expression encompassed jejunal morphological adaptations and consequently nutrient absorption. C) Gln+Glu consumption differences between diets do not account for distinct cell signaling events (50,51) or ornithine production (52). D) Arg and Met original content of MSP was higher than CAS, so its consumption by MSP-fed rats could lead to an efficient channeling for polyamine production (53). This also coincides with the good correlation found among ODC, AdometDC and SSAT gene expression in liver and Arg and/or Met consumption through out recovery.

In conclusion, casein and MSP, both good protein sources for cell physiology and renewal in liver and intestine, differ in their capabilities to enhance polyamine biosynthesis at transcriptional level for recovering of undernourished rats. The simultaneous study of mostly all members of this

metabolic pathway in this (or any) particular metabolic state helps to understand how cell adapts from ill to nourishment states, where different amounts of individual polyamines are needed. Even more, by using a battery of polyamine pathway genes, like the one used in this study, could improve the current knowledge of the role of polyamines in transfection studies (54) or cell restitution-compromised diseases like in small bowel resection (43).

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Table 1. Oligonucleotide sequences[1, 2]

Gene		Sequence (5'- 3')	Genbank Accession #	PCR Product
GAPDH	Fw	TAAAGGGCATCCTGGGCTACACT	NM017008	241
	Rv	TTACTCCTTGGAGGCCATGTAGG		
ODC	Fw	CATTCAGAGTTGACCTTGTGAGAG	M16982	915
	Rv	CTCAGCTATGATTCTCACTCCAGA		
AdometDC	Fw	TGAATGCTCTCTTCTGTGTTTAGG	M34464	998
	Rv	TGGTGCCATTAACTGTCAGAATAC		
SPdS	Fw	TCCTATTACCAGCTCATGAAGAC	AF337636	607
	Rv	AGACAGACACAGAGACTGAGTCC		
SPmS[2]	Fw	CAGGACCATGGMTATTTAGCAAC	NM009214[Mo]	877
	Rv	TGARAATTCCACAGGACAATACAG	BC009898[Hu]	
SSAT[2]	Fw	TCTTGARGACTTCTTCGTGATGAG	NM009121[Mo]	419
	Rv	GCAACAACGYCACTGGTAATAAAG	NM002970[Hu]	
OAZ	Fw	TTCAGCTTTCTTGGCTTTGAG	NM139081	242
	Rv	GTGTGACAAACACAGCATTCAG		
OAZI	Fw	CTACATGAATGACGGTGTTTATGG	D50734	400
	Rv	TTCAGTGGAGAAGTTGTCTTCTTG		

[1] Glyceraldehyde 3-phosphate dehidrogenase (GAPDH), Ornithine decarboxilase (ODC), *S*-adenosyl-methionine decarboxilase (SAMDC), Spermidine synthase (SPdS), Spermine synthase (SPmS), Spermidine/spermine-*N*1-acetyltransferase (SSAT), ODC antyzyme (OAZ), ODC antyzime inhibitor (OAZI).

[2] Fw = Forward, Rv = Reverse

[3] Rat gene sequence not available in GenBank, therefore the design was done based on mouse (Mo) and human (Hu) homologous sequences. R = A+G, Y = C+T, M = A+C

[4] Experimental size of SPmS and SSAT gene products as compared to DNA Standard (100-2000 bp Low mass DNA Ladder, Invitrogen, USA) in 1.5% agarose gel electrophoresis.

Table 2. Bioassay parameters during induction and recovery from rat malnutrition [1,2,3,4]

Group		Protein Intake (g)	Δ weight (g)	Energy (kcal)	Urea (g/100mL)	Arg Intake (g)	Met Intake (g)	Glx Intake (g)
Induction								
CAS 4%		4.6±0.6^{a}	8.4±2.3^{a}	417±57^{a}	15.4±.2.5^{a}	0.14±0.02^{a}	0.18±0.02^{a}	0.92±0.12^{a}
CAS 18%		44.2±2.9^{b}	112.7±9.3^{b}	961±6.3^{b}	30.0±4.6^{b}	1.33±0.09^{b}	1.72±0.11^{b}	8.8±0.58^{b}
Recovery								
1st Week	CAS18%	16.4±2.0^{c}	44.5±6.6^{c}	360±44^{c}	26.6±6.0^{b}	0.49±0.06^{c}	0.64±0.08^{c}	3.26±0.40^{c}
	MSP18%	16.2±0.9^{c}	47.1±3.1^{c}	384±22^{c}	17.4±3.5^{a}	1.04±0.06^{d}	0.83±0.05^{d}	3.06±0.17^{c}
2nd Week	CAS18%	33.5±3.6^{d}	79.2±7.5^{d}	734±78^{d}	22.1±8.3^{ab}	1.01±0.11^{d}	1.31±0.14^{e}	6.67±0.72^{d}
	MSP18%	33.9±1.1^{d}	79.6±6.4^{d}	802±27^{d}	17.8±4.5^{a}	2.17±0.07^{e}	1.70±0.06^{b}	6.41±0.21^{d}
3rd Week	CAS18%	41.2±5.9^{b}	108±8.8^{e}	1085±143^{e}	23.1±8.8^{ab}	1.24±0.18^{b}	1.61±0.23^{b}	8.20±1.17^{bd}
	MSP18%	44.1.±0.9^{b}	118±10.7^{e}	1248±37^{e}	19.0±8.5^{a}	2.82±0.06^{f}	2.25±0.05^{f}	8.33±0.17^{b}

[1] Average values from 4 rats per group.

[2] Values are expressed as mean ± SD; Cas = casein, MSP = modified soy protein, Glx = Glu+Gln

[3] Different superscripts letters within the same column means significant differences, $p<0.05$

[4] At less specified, all parameters during the induction period are end point values (21 days). All values expressed for recovery are cumulative at the corresponding period. Plasma urea corresponds to end point values at any stages of the experiment. Partial results were previously reported (25)

Table 3. Polyamine pathway mRNA level profiles in jejune during induction and recovery from rat malnutrition [1,2,3,4]

Group		ODC	AdometDC	SPdS	SPmS	SSAT	OAZ	OAZI
Induction								
CAS 4%		2.06±0.08^{a}	3.47±0.04^{a}	0.93±0.06^{a}	0.29±0.01^{a}	0.77±0.06^{a}	1.10±0.05^{a}	0.73±0.04^{a}
CAS 18%		2.63±0.26^{b}	3.98±0.36^{b}	1.13±0.03^{b}	0.44±0.01^{b}	1.00±0.02^{b}	1.33±0.03^{b}	0.96±0.02^{b}
Recovery								
1st Week	CAS18%	2.19±0.12^{a}	3.93±0.09^{b}	1.27±0.08^{c}	0.37±0.01^{c}	1.08±0.03^{c}	1.34±0.03^{b}	1.01±0.03^{bc}
	MSP18%	1.92±0.12^{a}	2.97±0.13^{c}	1.34±0.05^{c}	0.19±0.01^{d}	1.24±0.03^{d}	1.40±0.04^{b}	1.04±0.03^{c}
2nd Week	CAS18%	2.13±0.10^{a}	1.31±0.03^{d}	1.16±0.23^{abc}	0.32±0.02^{e}	1.02±0.02^{b}	1.14±0.02^{a}	0.92±0.03^{d}
	MSP18%	1.63±0.06^{c}	2.13±0.09^{e}	1.32±0.20^{bc}	0.13±0.01^{f}	1.06±0.04^{bc}	1.25±0.05^{c}	0.98±0.05^{bcd}
3rd Week	CAS18%	1.48±0.04^{d}	2.23±0.03^{e}	1.20±0.13^{bc}	0.31±0.01^{e}	1.03±0.04^{abc}	1.15±0.04^{a}	0.96±0.04^{b}
	MSP18%	1.59±0.04^{c}	2.45±0.04^{f}	1.20±0.13^{bc}	0.33±0.02^{e}	0.95±0.02^{d}	0.98±0.02^{d}	0.92±0.02^{d}

[1] Average mRNA values result from 4 replicates and related to a housekeeping gene (GAPDH) mRNA average values.

[2] Values are expressed as medium ± SD. Cas = casein, MSP =modified soy protein

[3] Different superscripts letters within a same column means significant differences, $p<0.05$

[4] See text for enzymes and regulatory proteins nomenclature

Table 4. Polyamine pathway mRNA level profile in ileum during induction and recovery from rat malnutrition [1,2,3,4]

Group		ODC	AdometDC	SPdS	SPmS	SSAT	OAZ	OAZI
Induction								
CAS 4%		1.19±0.03^{a}	2.94±0.27^{a}	0.85±0.02^{a}	0.82±0.01^{a}	1.68±0.12^{a}	1.30±0.06^{a}	0.89±0.02^{a}
CAS 18%		1.38±0.02^{b}	3.98±0.08^{b}	1.04±0.04^{b}	0.75±0.01^{b}	1.75±0.15^{a}	1.19±0.03^{b}	0.99±0.02^{b}
Recovery								
1st Week	CAS18%	2.94±0.10^{c}	4.24±0.20^{c}	0.86±0.03^{a}	0.67±0.05^{c}	1.87±0.09^{ab}	1.28±0.05^{a}	1.08±0.02^{c}
	MSP18%	2.74±0.07^{d}	1.91±0.04^{d}	0.88±0.02^{a}	0.89±0.05^{d}	2.05±0.14^{b}	1.34±0.05^{a}	1.30±0.04^{d}
2nd Week	CAS18%	2.77±0.31^{d}	1.75±0.06^{e}	0.70±0.02^{c}	0.73±0.04^{c}	1.73±0.11^{ac}	1.24±0.04^{ab}	1.15±0.02^{e}
	MSP18%	4.09±0.25^{e}	4.54±0.33^{c}	0.67±0.02^{c}	0.66±0.06^{c}	1.64±0.04^{c}	1.12±0.01^{c}	1.24±0.02^{f}
3rd Week	CAS18%	2.96±0.16^{cd}	3.39±0.27^{f}	0.66±0.03^{c}	0.72±0.07^{c}	1.79±0.06^{ac}	1.00±0.02^{d}	1.19±0.04^{ef}
	MSP18%	1.76±0.05^{e}	4.16±0.12^{bc}	0.81±0.05^{a}	0.83±0.04^{ac}	2.01±0.06^{b}	1.05±0.03^{d}	1.21±0.03^{f}

[1] Average mRNA level values result from 4 replicates and related to the housekeeping gene (GAPDH) mRNA average values.

[2] Values are expressed as medium ± SD. Cas= casein, MSP = modified soy protein

[3] Different superscripts letters within a same column means significant differences, $p<0.05$

[4] See text for enzymes and regulatory proteins nomenclature

Table 5. Polyamine pathway mRNA level profile in liver during induction and recovery from rat malnutrition[1, 2, 3, 4]

Group		ODC	AdometDC	SPdS	SPmS	SSAT	OAZ	OAZI
Induction								
CAS 4%		0.52±0.01^{a}	0.87±0.02^{a}	0.87±0.01^{a}	0.12±0.01^{a}	0.58±0.02^{a}	0.62±0.01^{a}	ND
CAS 18%		0.65±0.01^{b}	1.11±0.02^{b}	1.32±0.03^{b}	0.19±0.01^{b}	0.43±0.02^{b}	0.62±0.05^{a}	ND
Recovery								
1st Week	CAS18%	0.47±0.01^{c}	0.89±0.01^{a}	0.87±0.02^{a}	0.06±0.01^{c}	0.40±0.02^{b}	0.86±0.03^{b}	ND
	MSP18%	0.28±0.01^{d}	0.39±0.01^{c}	1.13±0.01^{c}	0.27±0.01^{d}	0.48±0.01^{c}	0.91±0.05^{bc}	ND
2nd Week	CAS18%	0.36±0.01^{e}	0.76±0.01^{d}	1.26±0.02^{d}	0.02±0.01^{e}	1.03±0.01^{d}	0.93±0.01^{c}	1.03±0.01^{a}
	MSP18%	0.31±0.01^{f}	0.69±0.01^{e}	0.79±0.01^{e}	0.25±0.01^{d}	0.52±0.02^{c}	0.75±0.03^{d}	1.14±0.05^{b}
3rd Week	CAS18%	0.56±0.01^{g}	0.84±0.01^{f}	0.85±0.02^{f}	0.34±0.01^{f}	0.65±0.02^{e}	1.19±0.01^{e}	0.70±0.03^{c}
	MSP18%	0.55±0.01^{g}	0.98±0.01^{g}	1.04±0.01^{g}	0.32±0.02^{f}	0.60±0.01^{a}	1.27±0.02^{f}	1.14±0.03^{b}

[1] Average mRNA level values result from 4 replicates and related to that housekeeping gene (GAPDH) average value.

[2] Values are expressed as medium± SD. Cas= casein, MSP =modified soy protein, ND =Not detected

[3] Different superscripts letters within a same column means significant differences, $p<0.05$

[4] See text for enzymes and regulatory proteins nomenclature

FIGURE LEGENDS

Figure 1 Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) and p53 in jejune of undernourished and control rats before nutritional rehabilitation. Control group (CAS18%) showed a stronger pattern of distribution of $PCNA^{+}$ cells at crypt (A) and $p53^{+}$ cell at villous tip and *Lamina propia* (C) than the CAS4% counterparts (B and C, respectively).

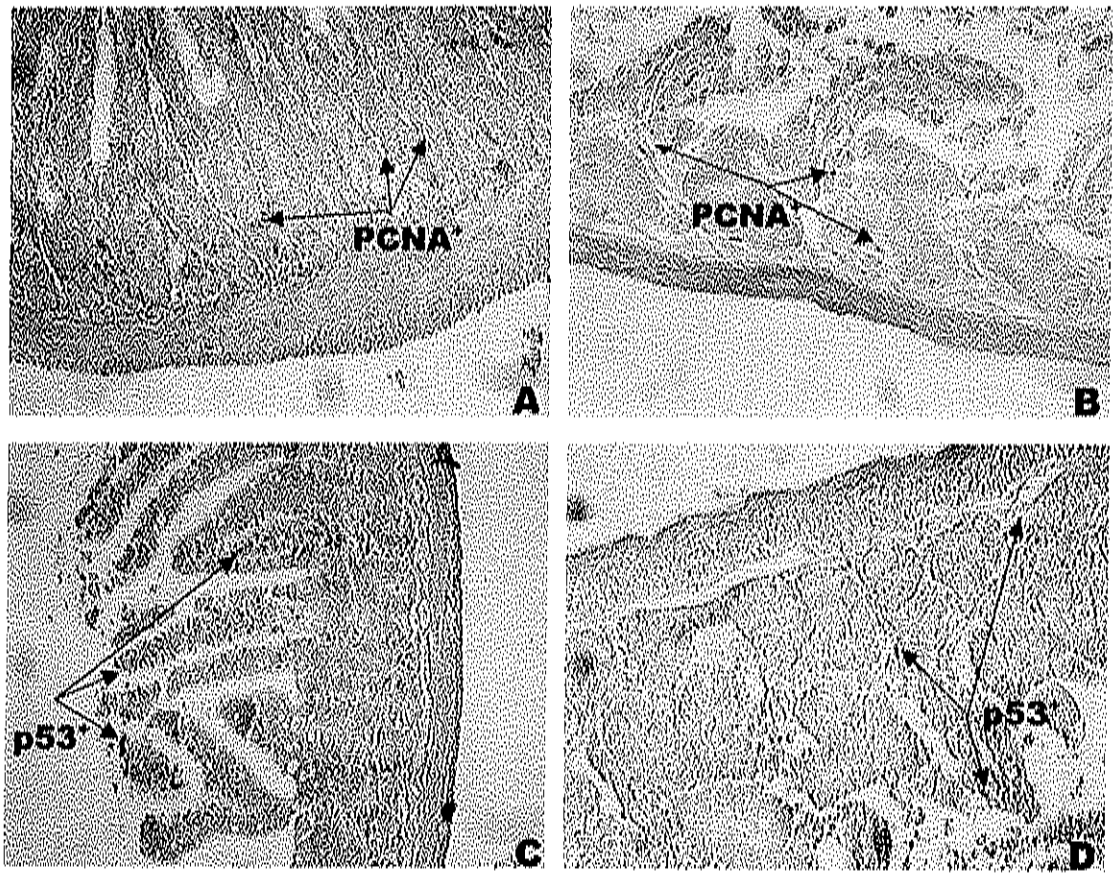

Figure 1

Liver and intestinal PPARα mRNA levels respond differently to protein intake and it is not related to SSAT and SPdS transcript levels in undernourished rats during recovery

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ABSTRACT

Background: Expression of the PPARα gene is influenced by diet and has been related to polyamine metabolism in starvation. **Objective**: to evaluate the hepatic and intestinal mRNA levels of PPARα, Spermidine/ spermine–N1-acetyltraferase (SSAT) and Spermidine synthase (SPdS) during rehabilitation of undernourished rats with two protein sources. **Methods**: 28 undernourished rats (fed 4% casein diet for 21 days) were fed for 21 days with two 18% protein diets: an enzymatically modified soy protein (MSP18%) and casein (CAS18%). mRNA levels of PPARα, SSAT and SPdS normalized against GAPDH were quantified by RT-PCR, before and after nutritional recovery. **Results**: Before recovery, jejunal but no hepatic or ileal PPARα mRNA levels increased when increasing protein content in diet. SPdS mRNA levels in all three tissues and intestinal SSAT transcripts also increased with protein content in diet. During recovery, PPARα transcript levels behave differently in each tissue, without significant differences between both diets at the end of the bioassay. There was no association between PPARα mRNA levels and those of SPdS or SSAT, independently of the diet and tissue. PPARα and SPdS correlated (r_s>0.5) with energy and protein intake in ileum. Although SSAT mRNA leves did not have direct relationships in ileum, positive correlations with energy and protein intakes were found for liver contrary to negative ones for jejune. **Conclusion**: PPARα mRNA levels depend on nutrient bioavailability, cellular recovery and is expressed in a tissue-specific manner, but is not related to SPdS and SSAT transcript levels during nutritional rehabilitation.

INTRODUCTION

Functional restoring of organs after malnutrition implies initially the replenishment of energy stores and then cellular proliferation. Different specific hormonal cascades before and after nutritional recovery (1) orchestrate the primary use of amino acids as fuel. Once energy demands are fulfilled, amino acids can follow other anabolic routes besides protein synthesis. Nutrient consumption for energy replenishment or for cell growth is also mediated by cell signaling (2) and gene transcription (3).

The peroxisome proliferator activated receptors (PPAR's) are nutrient–sensitive transcription factors responsible for energy providing substrate mobilization (4,1). The alpha isotype of PPAR (PPARα) is particularly sensitive to nutrient delivery in liver and intestine (5) and to stress conditions (6). PPARα is well known as an up-regulator of many genes related to lipid metabolism (7,8) and also been related to a down regulation of several enzymes of the urea cycle and polyamine biosynthesis during fasting (1).

On the other hand, polyamines are essential for cell proliferation and their depletion can cause growth arrest particularly in intestine mucosal cells. Therefore, PPARα may be involved in mechanisms responsible for switching amino acid metabolism (particularly Met and Arg), for cell growth and energy production. In fact, amino acids can stimulate, via Akt-mTOR signaling, transcription of the ornithine decarboxylase (ODC) gene (9,10), a rate limiting enzyme of the polyamine pathway. Also, human Spermidine/spermine-*N1*-acetyltransferase (SSAT), the main catabolic enzyme of the pathway, contains a PPAR-response element (PPRE) in its promoter which is activated by PPARγ in colon cancer cells (11). Since there is a tissue specific-differential expression of PPAR's, it is likely that SSAT transcription is also transcriptionally regulated by

PPARα in tissues like liver and small bowel, as it is in colon where PPARγ is mainly expressed (5).

To support the last cited hypothesis, Kernsten et al. (1), by using an oligonucleotide microarray, found that SSAT and Spermidine synthase (SPdS) gene expression were 3.2 and 2.7 times higher in fasted PPARα$^{-/-}$ mice than in PPARα$^{+/+}$ mice. Although, it is well known that fasting enhances glucocorticoid dependent-PPARα expression and activity (6) there are no reports about the consequences on transcriptional regulation of SSAT and SPdS genes in wild type (PPAR$^{+/+}$) mice in fed state. Even more, to our knowledge the influence of protein intake level or its origin (animal or vegetable) in PPARα mRNA levels, has not been reported. Thus, in this study we investigated the possible relationship among PPARα, SSAT and SPdS gene expression in response to dietary protein (casein 4% and 18%) before and after nutritional rehabilitation of undernourished rats fed two high quality proteins of different origin.

METHODS

Animals and tissues. To evaluate the effect of the level and type of dietary protein administration in undernourished Wistar rats, 28 rats (21 days old) were fed a 4% casein diet for 21 days. Four of these rats and 4 fed an 18% casein-based diet for the same period, were anesthetised by intraperitoneal injection of 6.25 mg/kg of body weight of tiletamine-HCl and zolazepam-HCl and sacrificed at the 21st day. Two groups of 12 rats were then re-fed with 18% protein diets based on modified soy protein (MSP) and casein for 21 days . Four rats of each group were anesthetised and killed at day 7,14 and 21. MSP characteristics, isoenergetic diet formulations and feeding protocol have been recently reported (12). All animals were supplied by the local inbreed colony of our institute. Both experiments were performed after approval of an internal ethical committee. Body weight and food consumption was recorded daily to estimate bioassay parameters.

All anesthetised rats underwent the same surgical procedures. First, as soon as the abdominal cavity and thorax were opened, blood was collected in tubes containing EDTA-K$^+$ by intra-cardiac puncture. The intestine was rapidly excised from the ligament of Treitz to the ileocecal valve and rinsed with saline to eliminate excess of blood. Ileal and jejunal segments were identified at 2 and 30 cm from the ileocecal valve. Two portions of 1 cm from every segment were cut, were placed in RNase- free sterile plastic tubes and frozen in liquid nitrogen. This procedure was also applied to portions of liver (300 mg). Plasma was separated from whole blood by centrifugation at 1,000 x *g*, and its urea content measured with a Urease-GLDH commercial kit (SPINREACT, S.A., Girona, España). Immediately, after blood collection and organs removal, the animal was killed by cervical dislocation. All biological samples were kept at –70°C until analysis.

Isolation of liver and intestinal RNA. Total RNA was prepared from individual frozen tissues using Trizol™ reagent (GIBCO BRL, Burlington, Canada) from 100 mg and 200 mg of liver and intestine respectively, according to manufacturer instructions. The quality and quantity of RNA (resuspended in DEPC treated water) was evaluated espectrophotometrically at 260 and 280 nm. RNA Integrity was confirmed using a 1% agarose-formaldehyde gel electrophoresis to analyze 5 µg of denatured total RNA, stained with ethidium bromide and visual observation of ribosomal RNA bands under UV. After quality and integrity confirmation, 4 RNA samples (20 µg of each sample) were pooled per tissue (ileum, jejune and liver) and experimental treatment (mentioned in animals and tissues section).

Semiquantitative RT-PCR. cDNA was obtained from 15 µg of each pooled total RNA from liver or intestinal RNA-sample using Superscript™ first-strand synthesis system (Invitrogen, Carlsbad, CA) following the manufacturer instructions, in a total volume of 60 µL. Controls for gDNA contamination (-RT) and a control mRNA (+RT) were included. PCR amplifications were carried out using specific set of primers for PPARα, SPdS, SSAT and GAPDH (**Table 1**) in a total volume of 50 µL as follows: 6 µL of cDNA (1.5 µg from total RNA), 30.5 µL water, 5.6 µL $MgCl_2$ (25mM), 4.2 µL PCR buffer 10X, (50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.001% gelatin), 1.4 µL dNTP mix (dATP, dTTP, dGTP, dCTP, 10 mM each), 2µL primer mix (20 µM Forward: 20 µM Reverse), and 2.5 U *Taq* Polymerase (SIGMA, St. Louis MI).

GADPH mRNA amplification was used to optimize the PCR reaction, since higher mRNA levels of this constitutive gene were expected. Conditions to reach a linear PCR amplification of the GAPDH gene were determined at two different cDNA concentrations (1.5 and 3 µg of total RNA). The optimized conditions were: 85°C,30 sec; 94°C, 3 min; followed by 35 cycles of 94°C, 30

sec; 60°C, 30 sec; 68°C, 30 sec. PCR products were then separated by 2% agarose gel electrophoresis and stained with ethidium bromide. Gel image was obtained and analyzed using Digital Science ID software (Kodak, Rochester NY) and the total band net intensity evaluated by densitometry. Relative concentration (pixels) of target gene to GADPH gene were then calculated for each set of genes and treatments.

Statistics. Values are expressed as means ± SE. Comparisons between means were done by ANOVA (13) and Tukey Kramer comparison test. Spearman correlation rank (r_S) test was also performed between dietary factors (energy and protein consumption) with mRNA levels. All statistical analyses were considered to be significant at $p < 0.05$ level.

RESULTS

Biological response to diets.

At the end of the malnutrition induction period (21 days), 4% casein-fed rats (CAS4%) gained 13.4 times less body weight than controls (CAS18%). Protein consumption of CAS4%-fed rats was 1/10 that observed for the CAS18%-group, although both groups used protein for sustain growth in the same extent. The low protein diet consumption also resulted in a 50% lower serum urea level in CAS4% *vs.* CAS 18%-fed rats. Also, the CAS4% group consumed 50% of the energy consumed by the CAS18% group

mRNA levels of PPARα, SPdS and SSAT.

PPARα mRNA accumulation (normalized to GAPDH values) in liver, jejune and ileum before and after nutritional recovery are shown in **Figure 1**. There was a significant decrease ($p<0.05$) in hepatic PPARα mRNAlevels between rats fed the 4% *vs.* 18% casein-diet in liver (0.39 *vs.* 0.31) and ileal (0.44 vs. 0.11) but an increase in jejunal PPARα mRNA level (0 vs. 0.22). However, during recovery with MSP18% and CAS18% diets, although there was a tissue-specific responsiveness on PPARα mRNA levels (liver>jejune>ileum), there was no differential effect between both diets, except for the 1st week. In liver and jejune PPARα transcripts had a two peak response (1 and 3rd. week) while in ileum, it was almost maintained throughout the recovery period. At the end of the recovery period (3rd week), PPARα mRNA levels were equally stimulated in the three tissues with both diets.

During the malnutrition period (animals fed the 4% and controls), the SPdS mRNA steady state levels followed a PPARα- like pattern in jejune, while the same effect was observed for SSAT and PPARα in liver and jejune (**Figure 2**). Conversely, during nutritional recovery, there were no association between

PPARα or any of the SPdS or SSAT mRNAs, independently of the sumministered diet and tissue. However, significant correlations were found between PPARα and energy or protein intakes for ileum (**Table 2**), and a similar association with both dietary parameters was found for SPdS in the same tissue . Although SSAT mRNA level have no relationship with dietary parameters in ileum, positive correlations with energy and protein intakes were found for liver and negative for jejune (**Table 2).**

DISCUSSION

The peroxisome proliferator activated receptor alpha (PPARα) activity is implicated in energy metabolism and cell growth (3). Cellular decisions for growth or energy replenishment rely in PPAR action in a tissue specific manner (14), although there are other metabolic adaptations (2). Advantageously, the same hormonal stimulus that mediates metabolic adaptations to low energy status also stimulates PPARα gene transcription, mRNA and protein stabilisation by reducing its degradation (15). Once metabolic demands are fulfilled, the cell returns to growth coinciding with growth factors- mediated signalling, reduced ligand activation and PPARα protein degradation (16). Therefore, for the study of metabolic adaptations processes of organs to undernutrition, it is important to evaluate them at the gene transcription level (17).

Up regulation of the PPARα gene occurs during starvation (4) which in turn can be efficiently activated by serum fatty acid released from adipose tissue in response to hormonal stimuli (8). Among organs, intestine and liver are the most sensitive tissues to starvation (5) which also coincides with its low cell turnover rate and PPARα presence (7). However, PPARα gene expression had not been previously evaluated in response to protein intake level in normal nor in protein-energy malnutrition states. In this study, we found that long term administration of low (4%) and high (18%) protein diets to undernourished young rats, resulted in a differential PPARα expression pattern. Although starvation induces a simultaneous up regulation of PPARα gene expression in hepatic and intestinal tissues, this phenomenon was found only in ileum and liver but not in jejune in response to a low level of dietary protein. One of the most important adaptations of the starved intestine is the increase of brush border transporters to attain the maximal absorption of nutrients (18,19). There is also a high

catabolism of nutrients in intestinal mucosa (20) which is increased in stressed conditions. Additionally, we have previously seen that in well nourished rats the maximal absorption of nutrients is at jejunal level (data not shown).

Therefore, it seems likely that liver and ileum follow an starvation-like behaviour resulted from the low bio-disponibility of nutrients as consequence of the high jejunal nutrient consumption (**Figure 3A**). During this stage, jejune uses luminal nutrients as a fuel while liver and ileum uses body fuel storage (mainly fatty acids). Since transport mechanisms are efficient in jejune, PPARα is not as useful in jejune as it is in liver and ileum where, other extracelullar hormonal stimuli or intracellular signalling may occur. Increasing dietary protein load result in an efficient nutrient uptake in jejune where they are rapidly degraded remaining only real small quantities for ileum absorption (**Figure 3B**). During jejune healing there is a strict balance between nutrient consumption used for growth or as fuel. Thus, the surplus nutrients, particularly amino acids, distributed to other organs including liver and ileum, depends on jejunal cell restitution (**Figure 3C**). Once jejune energy and growth demands are fulfilled, all other organs progressively switch nutrient fate to fuel and use them for growth.

Spernidine/spermine-*N1*-acetyltranferase (SSAT) and Spermidine synthase (SPdS) gene expression in PPAR-null mice (PPARα$^{-/-}$) were 3.2 and 2.7 times higher than in the wild-type (PPARα$^{+/+}$) counterpart in fasting state (1), but the values of PPARα gene expression during feeding neither in wild type nor null mice. Malnutrition triggers a special scenario for PPARα action (high serum glucocorticoids, high free fatty acid in serum and PPAR gene up regulation). Although differences in PPAR expression patterns among tissues was observed, PPARα mRNA levels were inverse to those observed for SSAT and SPdS genes in jejune in response to a low protein intake. Once again, it is possible that the maximum utilization of nutrients in jejune, compromised the relation of

PPARα mRNA levels with those of the polyamine pathway. However, this relationship was completely lost upon nutritional rehabilitation.

Moreover, the origin of supplied protein, which differed in amino acid composition (particularly Arg and Met) and molecular form (intact vs. hydrolysate), did not elicit a differential PPARα gene expression. Particularly, soy protein administration produces a differential up-regulation of many genes involved in lipid metabolism and cell growth (21). However, the presence of some anti-physiological compounds limits its absorption, so gene transcription consequences can resemble that of low quality proteins (22). The fact that both diets tend to have the same effect on gene expression, particularly adds a new argument about a comparable nutritional quality of protein modification (MSP) of vegetable proteins with animal intact proteins (casein), as we previously tested (12).

In conclusion, the marginal consumption of protein, as it was observed in protein energy malnutrition (PEM), affects PPARα levels in response to dietary protein level but it is conditioned to an efficient nutrient delivery. In addition, PPARα gene expression did not correlate with that of the SSAT and SPdS in the tissues tested. Therefore, it seems likely that other metabolic processes at the protein level (22) can impact either mRNA transcription or stabilization of the SPdS and SSAT gene expression irrespective of the tissue. More specific studies are needed to relate this co-expression in normal and pathological conditions like malnutrition.

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Table 1. Oligonucleotide sequences[1,2]

Gene		Sequence (5´- 3´)	Genbank Accesion #	PCR Product bp
GAPDH	Fw	TAAAGGGCATCCTGGGCTACACT	NM 017008	241
	Rv	TTACTCCTTGGAGGCCATGTAGG		
SPdS	Fw	TCCTATTACCAGCTCATGAAGAC	AF337636	532
	Rv	AGACAGACACAGAGACTGAGTCC		
PPARα	Fw	CTGAACATCGAGTGTCGAATATG	M88592	806
	Rv	TTGAACTTCATAGCGAAGTCAAAC		
SSAT[3,4]	Fw	TCTTGARGACTTCTTCGTGATGAG	-	419
	Rv	GCAACAACGYCACTGGTAATAAAG		

[1] Glyceraldehyde 3-phosphate dehydrogenase, Spermidine synthase (SPdS), Peroxisome Proliferator Activated Receptor Alpha (PPARα), Spermidine/spermine-*N1*-acetyltransferase (SSAT).

[2] Fw = Forward, Rv =Reverse

[3] Rat gene sequence not available in Genbank, therefore the design was done based on mouse (NM 009214) and human (NM 009121) homologous sequences. R = A+G, Y= C+T.

[4] Experimental size of SSAT fragment obtained by comparison with a DNA Standard (100-2000 bp Low mass DNA Ladder, Invitrogen , USA) in 1.5% agarose gel electrophoresis.

Table 2. Spearman correlation rank (r_S) between mRNA levels and dietary intakes, during rehabilitation [1,2].

Gene		Energy Intake	Protein Intake
PPARα	Liver	-0.09	0.09
	Jejune	0.14	0.26
	Ileum	0.81	0.90
SPdS	Liver	-0.26	-0.37
	Jejune	-0.41	-0.52
	Ileum	-0.60	-0.66
SSAT	Liver	0.60	0.54
	Jejune	-0.77	-0.83
	Ileum	-0.14	-0.26

[1] Peroxisome, proliferator activated receptor alpha (PPARα), Spermidine synthase (SPdS), Spermidine/spermine-n1-acetyl transferase (SSAT).

[2] $p<0.05$

FIGURE LEGENDS

Figure 1. PPARα mRNA levels in liver, ileum and jejune before and after nutritional recovery of undernourished rats. Different superscripts for the same tissue mean significant statististical differences ($p<0.05$). Malnutrition iinduction period (4% and 18% groups) compared to 18% casein (CAS)- and 18% modified soy protein (MSP)- fed rats after 1 week (CAS1,MSP1), 2 weeks (CAS2, MSP2) and three weeks (CAS3, MSP3) of nutritional recovery.

Figure 2. Hepatic (A), jejunal (B) and ileal (C) PPARα, SPdS and SSAT mRNA levels in response to low (4%) and high (18%) dietary protein level, before nutritional rehabilitation of undernourished rats.

Figure 3. Hypothetical model of inter organ regulation of PPARα mRNA levels by nutrient bioavailability during rehabilitation of undernourished rats. (A) Protein malnutrition induces morphological changes but increases nutrient absorption and utilization in jejune for energy production. Jejune metabolism during this period limits luminal and systemic delivery of nutrients to ileum and intestine respectively. This result in a differential expression pattern of PPARα mRNA levels in the three tissues. (B-C) Surplus amino acid delivery promotes increments in jejune cell mass and nutrient delivery in lumen and blood in a step-by-step fashion. The last results in a differential switching between protein use as fuel or for cell growth. Small arrows = PPARα mRNA levels, Dotted and wide arrows = Nutrient flow.

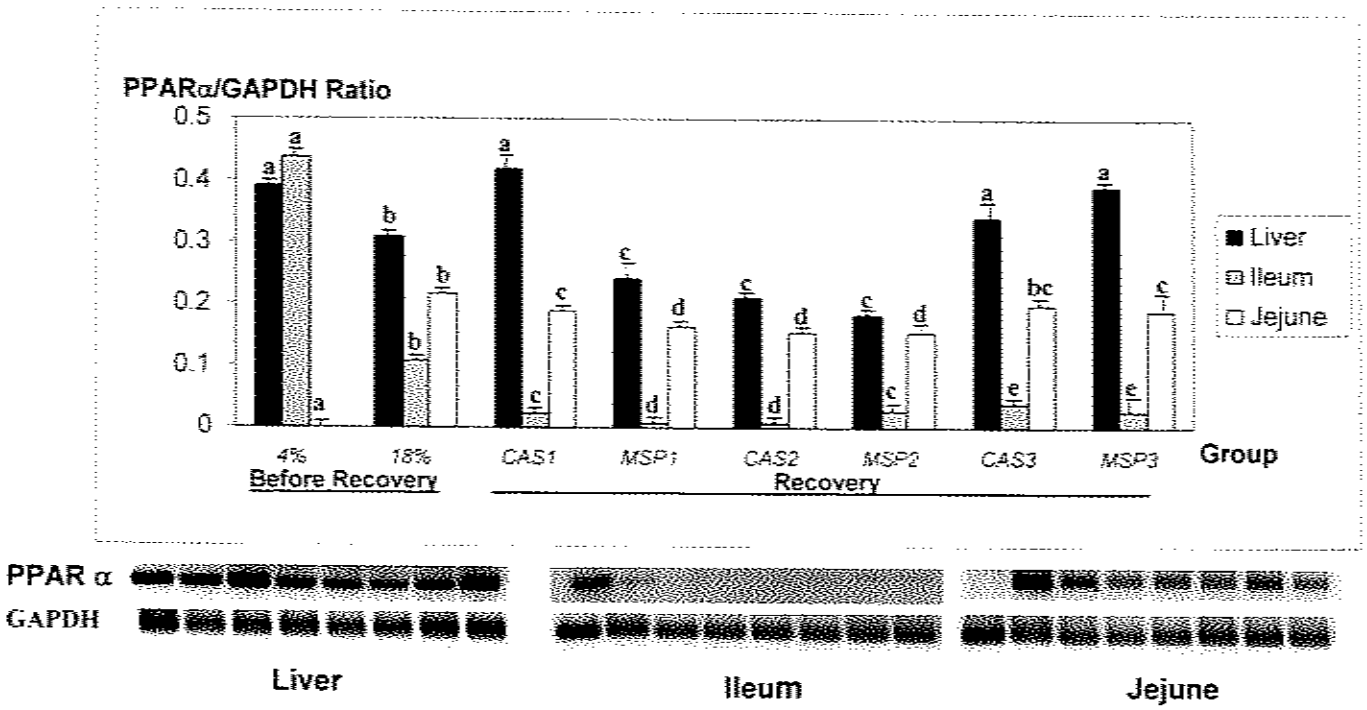
PPARα/GAPDH Ratio
0.5
0.4
0.3
0.2
0.1
0
Liver
Ileum
Jejune
4%
18%
CAS1
MSP1
CAS2
MSP2
CAS3
MSP3
Group
Before Recovery
Recovery
PPAR α
GAPDH
Liver
Ileum
Jejune

Figure 1

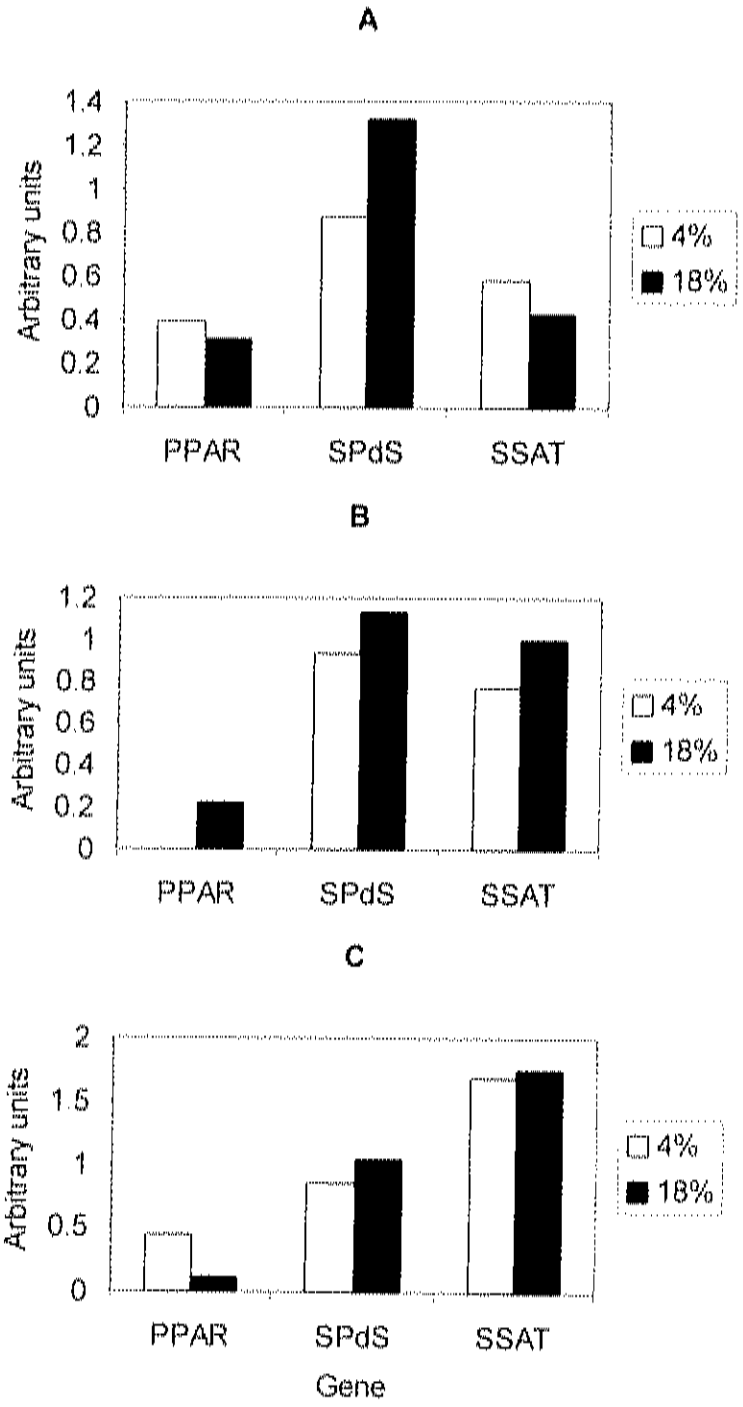
A
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Arbitrary units
PPAR
SPdS
SSAT
4%
18%
B
1.2
1
0.8
0.6
0.4
0.2
0
Arbitrary units
PPAR
SPdS
SSAT
4%
18%
C
2
1.5
1
0.5
0
Arbitrary units
PPAR
SPdS
SSAT
Gene
4%
18%

Figure 2

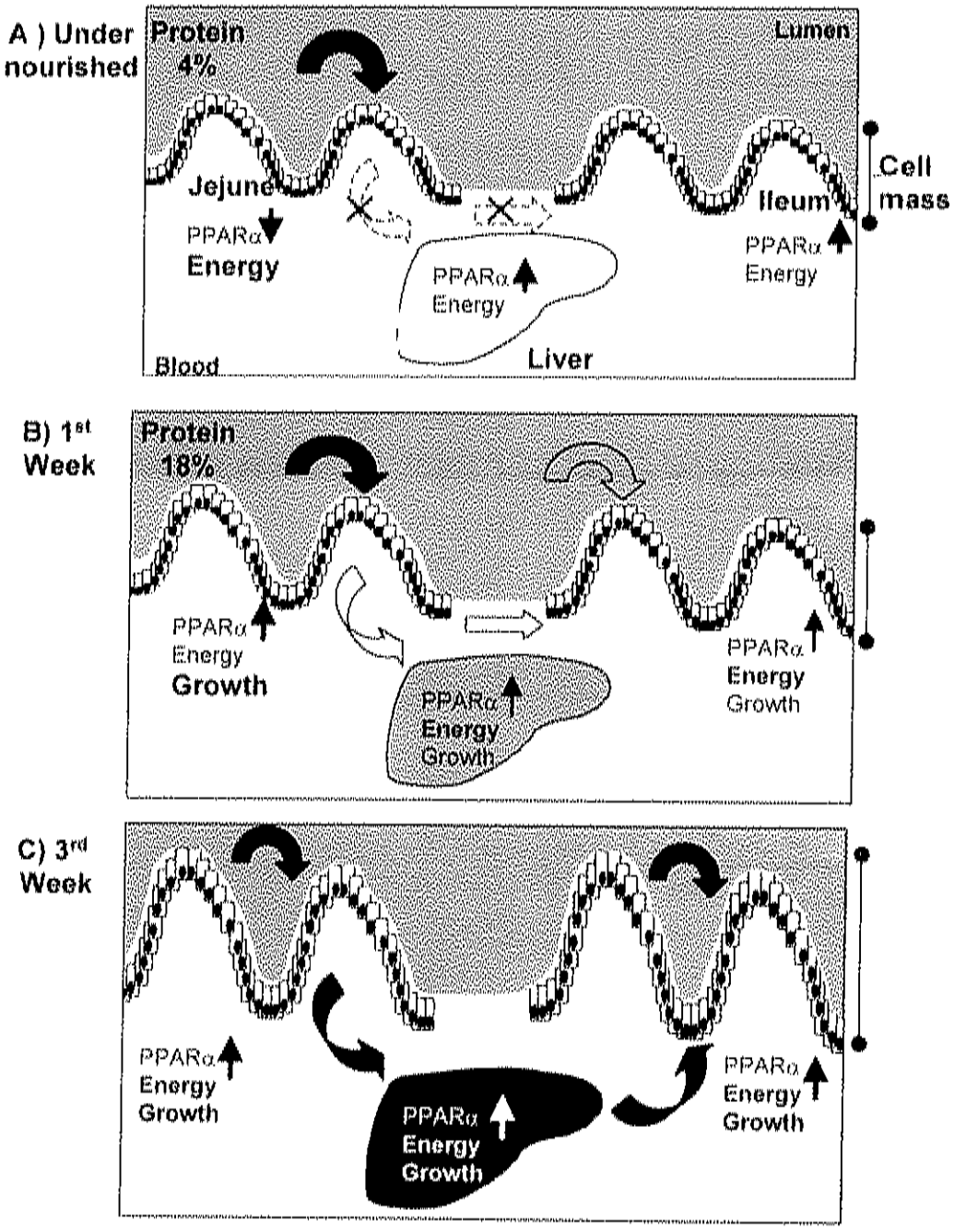
A) Under nourished
Protein 4%
Lumen
Cell mass
Jejunum
Ileum
PPARα
Energy
PPARα
Energy
PPARα
Energy
Blood
Liver
B) 1st Week
Protein 18%
PPARα
Energy
Growth
PPARα
Energy
Growth
PPARα
Energy
Growth
C) 3rd Week
PPARα
Energy
Growth
PPARα
Energy
Growth
PPARα
Energy
Growth

Figure 3

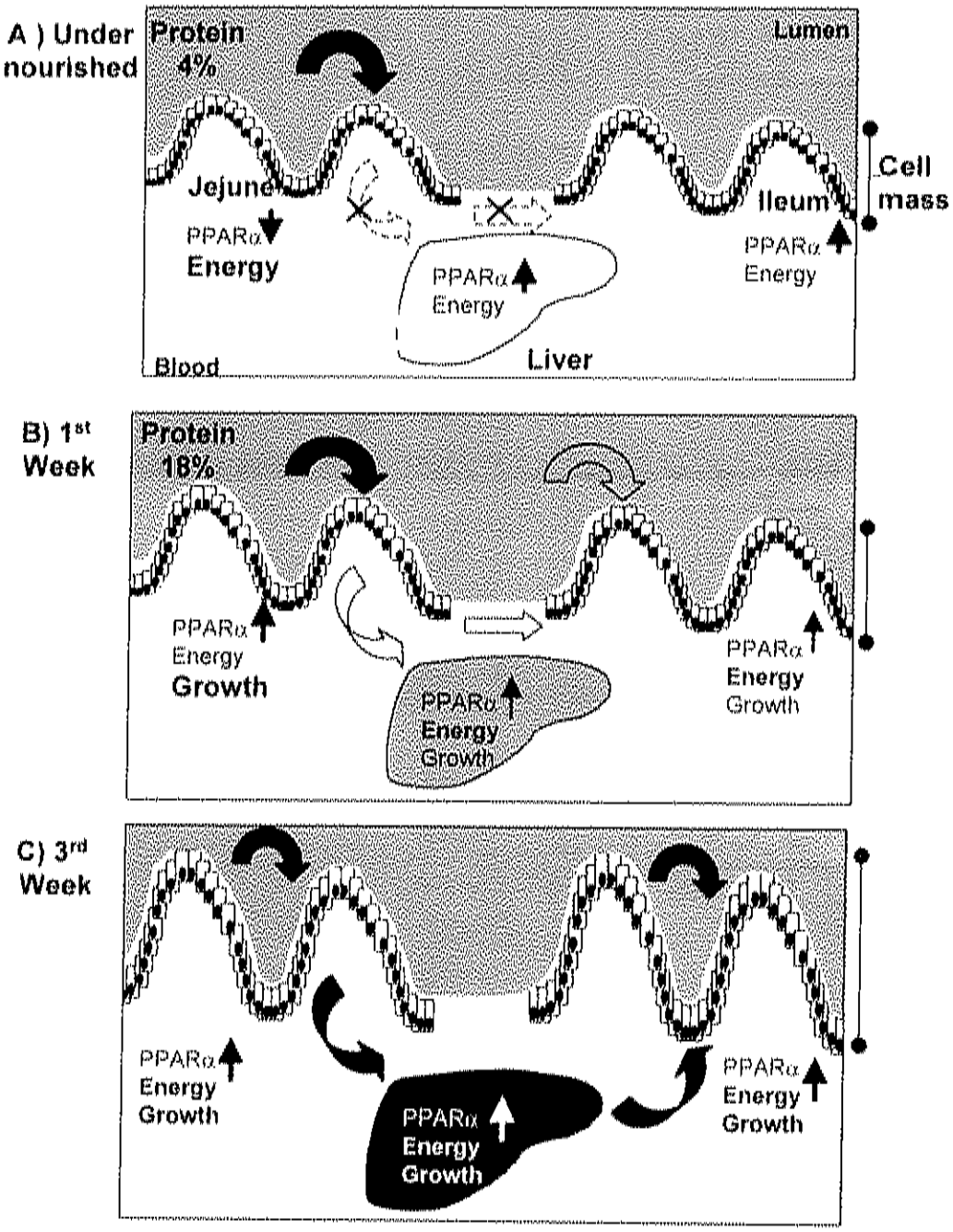
A) Under nourished
Protein 4%
Lumen
Jejunum
Ileum
Cell mass
PPARα
Energy
PPARα
Energy
PPARα
Energy
Blood
Liver
B) 1st Week
Protein 18%
PPARα
Energy
Growth
PPARα
Energy
Growth
PPARα
Energy
Growth
C) 3rd Week
PPARα
Energy
Growth
PPARα
Energy
Growth
PPARα
Energy
Growth

Figure 3